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Article *in* Plant Growth Regulation · November 2000

DOI: 10.1023/A:1010718020095 · Source: OAI

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Plant Growth Regulation **32:** 193–203, 2000.

Long-distance signalling and a mutational analysis of branching in pea

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Key words: apical dominance, auxin, branching, cytokinin, *Pisum*, *ramosus* mutants

Abstract

Four *ramosus* mutants with increased branching at basal and aerial nodes have been used to investigate the genetic regulation of bud outgrowth in *Pisum sativum* L. (garden pea). Studies of long-distance signalling, xylem sap cytokinin concentrations, shoot auxin level, auxin transport and auxin response are discussed. A model of branching control is presented that encompasses two graft-transmissible signals in addition to auxin and cytokinin. Mutants *rms1* through *rms4* are not deficient in indole-3-acetic acid (IAA) or in the basipetal transport of this hormone. Three of the four mutants, *rms1*, *rms3* and *rms4*, have very reduced cytokinin concentrations in xylem sap from roots. This reduction in xylem sap cytokinin concentration appears to be caused by a property of the shoot and may be part of a feedback mechanism induced by an aspect of bud outgrowth. The shoot-to-root feedback signal is unlikely to be auxin itself, as auxin levels and transport are not correlated with xylem sap cytokinin concentrations in various intact and grafted mutant and wild-type plants. *Rms1* and *Rms2* act in shoot and rootstock to regulate the level or transport of graft-transmissible signals. Various grafting studies and double mutant analyses have associated *Rms2* with the regulation of the shoot-to-root feedback signal. *Rms1* is associated with a second unknown graft-transmissible signal that is postulated to move in the direction of root-to-shoot. Exogenous auxin appears to interact with both of the signals regulated by *Rms1* and *Rms2* in the inhibition of branching after decapitation. The action of *Rms3* and *Rms4* is less apparent at this stage, although both appear to act largely in the shoot.

1. Introduction

A mutant-based approach toward understanding axillary bud outgrowth in plants has been undertaken for a number of species including *Arabidopsis*, tomato, petunia and garden pea. Mutations causing increased or decreased branching include the *axr* series from *Arabidopsis* [e.g., 23]; *bu*, *ls* and *to-2* from tomato [e.g., 14, 35]; the *dad* series from petunia [e.g., 28] and *bushy* [43] and the *ramosus* series from pea [e.g., 28]. At present, the *ramosus* mutants of pea (*Pisum sativum* L.; e.g., Figure 1) represent the largest range of phenotypically and physiologically described, relatively non-pleiotropic, increased branching mutants [1, 2, 6–10, 28, 31, 39, 42]. Mutants *rms1* through to *rms5* display increased branching at basal and aerial nodes [2, 31, 42].

The aim of this review is to present the simplest explanation for the control of branching in pea, based on physiological and genetic analyses. In so doing, this review presents the most detailed genetic model of the regulation of axillary bud outgrowth in higher plants. The model is developed mostly from previous studies with the *ramosus* mutants *rms1* through to *rms4* [6–10, 28, 38], but also incorporates new data on xylem sap cytokinin concentrations in intact and grafted plants, auxin response and auxin transport. Grafting studies with mutant and wild-type (WT) plants have been used to elucidate sites of gene action and to demonstrate roles for long-distance signals.

Figure 1. Phenotype of 54-d-old WT (Torsdag), *rms2-1*, *rms3-2* and *rms4-1* plants grown under a natural photoperiod of about 12 h.

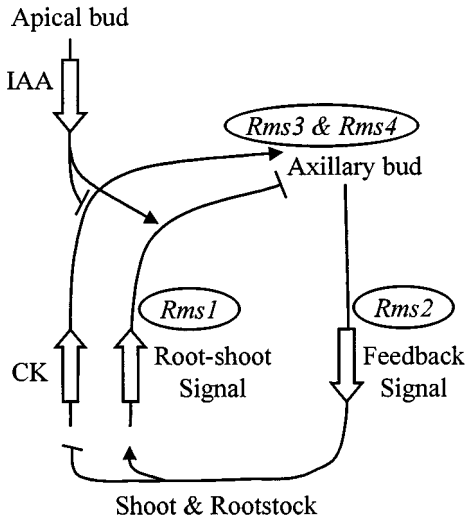

Figure 2. Working model of long-distance signalling among the apical bud, axillary bud(s) and the remainder of the plant (shoot & rootstock) together with the sites of action of the *Ramosus* genes *Rms1* through *Rms4* in pea. *Rms1* and *Rms2* act in shoot and rootstock. *Rms3* and *Rms4* are shown associated directly with axillary buds based only on data indicating they act mostly in the shoot. Large unfilled arrows indicate direction of signal movement; line arrowheads indicate promotion; flat ended lines indicate inhibition. The root-shoot signal regulated by *Rms1* is shown as a branching inhibitor, but results to date are also consistent with *Rms1* down-regulating level of a promoter. CK, cytokinin; IAA, indole-3-acetic acid.

These results, coupled with endogenous and exogenous hormone studies, have led to the placement of cytokinin and auxin in a working model that also encompasses two unknown graft-transmissible signals (Figure 2).

2. The *Ramosus* system in pea

Pea architecture is such that axillary buds may remain inhibited throughout ontogeny (Figure 1; WT). Bud release and subsequent growth (Figure 1; *rms2*, *rms3*, *rms4*) may be readily promoted in juvenile and adult plants and is not tightly constrained by the transition to flowering [see review by 28]. Therefore, most processes involved in flowering and/or branching can be readily distinguished in pea. Long-distance signalling can easily be monitored in juvenile and mature shoots through grafting, measurement of xylem sap constituents, and radiolabelled hormone transport studies. Studies with the *ramosus* mutants have also generally enabled branching to be investigated without the requirement of decapitation.

Over 30 mutants with increased branching at basal and aerial nodes define five *ramosus* loci [1, 2, 31, 42]. This large number of alleles indicates that further mutagenesis in WT pea is unlikely to identify many new mutants with a similar phenotype that correspond to new *ramosus* loci. Although it is likely that additional genes control bud outgrowth in pea, there are several reasons why they could be difficult to obtain in mutant screenings. Firstly, they could be involved in overlapping or redundant pathways, compensated for by homeostatic regulation duplicated in the plant genome. Secondly, a mutation in those genes might not cause phenotypes sufficiently distinct to enable isolation in a screen for increased branching phenotypes. Thirdly, the genes might reside in regions of the genome that are not readily mutated or the mutations might be lethal. Finally, a mutational analysis is very unlikely to reveal polygenic regulation of branching. In contrast, many mutations may

lead to bud outgrowth as part of a highly pleiotropic phenotype.

3. *Ramosus* genes differ in sites of action

Grafting studies have shown that *Rms1* and *Rms2* act in the rootstock and shoot to control the level or transport of graft-transmissible substance(s) (Table 1; [7, 9]). This is clearly demonstrated by the observation that branching (bud release and subsequent outgrowth) in *rms1* and *rms2* scions is inhibited by grafting to WT rootstocks [7, 9]. The *Rms1* and *Rms2* genes must also act in the shoot because branching is fully inhibited in WT scions grafted to mutant rootstocks. To date, grafting studies with *ramosus* mutants have utilised an epicotyl/epicotyl grafting technique [7] and the rootstocks have therefore included epicotyl tissue, cotyledons and roots. Although it is clear that *Rms1* and *Rms2* gene action occurs in the shoot (scion), it is not known whether gene action in the rootstock is located in the roots, cotyledons, epicotyl, or some combination of these. It also remains unknown whether *rms1* and *rms2* alter synthesis, metabolism, conjugation, transport or compartmentalisation of translocatable substance(s). A possible interaction between *Rms1* and *Rms2* is discussed later.

In contrast with *Rms1* and *Rms2*, the *Rms3* and *Rms4* genes may control bud release by action predominantly in the shoot (Table 1, [8]). Bud release in *rms3* and *rms4* scions is not reduced by grafting to rootstocks of other genotypes. Initial grafting studies with *rms3* plants carrying the *rms3-2* allele indicated that *Rms3* may affect the level or transport of a graft-transmissible substance because lateral growth after bud release was reduced in *rms3-2* scions by grafting with WT rootstocks [8]. However, as with grafting studies with *rms4* scions, this effect on lateral shoot growth was not repeated in reciprocal grafting studies with three other *rms3* alleles: *rms3-1*, *rms3-4* and *rms3-5* (R Floyd, I Murfet, P Walton, S Morris, C Rameau and C Beveridge, unpublished results). Consequently, in the model presented herein (Figure 2), *Rms3* and *Rms4* have been grouped together as acting in the shoot, perhaps in axillary buds.

Grafting studies indicate that *rms3* and *rms4* rootstocks do not prevent action of *Rms1* and *Rms2* genes in the rootstock as these mutant rootstocks inhibit bud release in *rms1* and *rms2* scions to a similar (or greater) extent than do WT rootstocks (Table 1, [8, 9]).

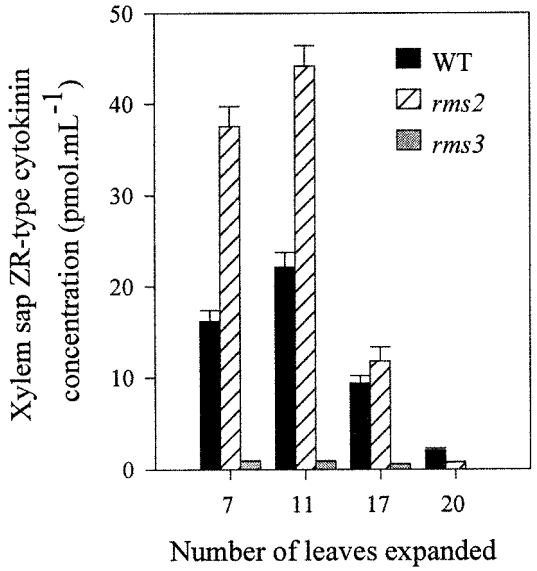

Figure 3. ZR-type cytokinin concentration in the xylem sap from the roots of WT (Torsdag), *rms2-1* and *rms3-2* plants at various ontogenetic stages. Xylem sap was harvested using a syringe-suction method and cytokinins from individual plants analysed by ELISA as described by Beveridge et al. [6]. All plants were harvested on the same day and the node of flower initiation was 17 or 18. Data are presented as mean + SE; n = 9 or 10. Photoperiod 16 h.

4. Relationship between branching and root xylem sap cytokinin concentration

4.1 *The roots of three* ramosus *mutants have low xylem sap cytokinin concentrations*

At most, if not all stages of ontogeny, mutant *rms1*, *rms3* and *rms4* roots have substantially lower concentrations of cytokinin in the xylem sap compared with WT roots (Figure 3, Table 1, [6, 7]). In contrast, the xylem sap cytokinin concentration in *rms2* roots appears to be slightly elevated compared with that of WT plants (Figure 3; [9]).

The possible cause(s) and consequence(s) of the altered cytokinin concentrations in *ramosus* mutant xylem sap, particularly the reduced levels in *rms1*, *rms3* and *rms4* plants, are quite intriguing. Despite the up to 80-fold difference in ZR concentration in the xylem sap of *rms1*, *rms3* and *rms4* plants compared with *rms2* plants, there are few obvious phenotypic differences among the mutant shoots. Nevertheless, under some conditions, small phenotypic differences are apparent that might be cytokinin-related. Different mutant *rms2* and *rms4* alleles cause leaves of intact mature plants to senesce several days later and earlier, respectively, than those of comparable WT plants [3;

Table 1. Summary of physiology of *ramosus* mutants *rms1* through *rms4*. A tick (✓) denotes branching in scions grafted to given WT and mutant rootstocks. Similarly, in comparison with intact WT plants, a tick denotes elevated shoot auxin levels, reduced basipetal auxin transport, reduced response to exogenous auxin applied to the decapitated stump and for reduced xylem sap cytokinin concentrations in mutant plants. A cross (×) refers to near WT values. Data extrapolated from [6–10], Figures 3 and 6 and Table 2

		Shoot genotype				
		WT	*rms1*	*rms2*	*rms3*	*rms4*
Highly branched with:	WT rootstock	×	×	×	✓	✓
	rms1 rootstock	×	✓	✓	✓	✓
	rms2 rootstock	×	×[a]	✓	✓	✓
	rms3 rootstock	×	×	×	✓	✓
	rms4 rootstock	×	×	×	✓	✓
Elevated shoot auxin level		×	✓	✓	✓	×
Reduced basipetal auxin transport		×	×	×	×	×
Reduced response to auxin		×	✓	✓	✓	✓
Reduced xylem sap cytokinin content		×	✓	×	✓	✓

[a] branching phenotype intermediate between *rms1*/WT and *rms1/rms1* plants (scion/rootstock).

see also Figure 1 in 7]. Compared with WT plants, young leaves of mutant *rms2-1* and *rms2-2* plants tend to wilt temporarily when exposed to a drying atmosphere [16].

Studies are underway to determine whether the lack of major phenotypic aberrations in *rms1*, *rms3* and *rms4* shoots is because the large reductions in concentration of root xylem sap cytokinin are not paralleled by similar reductions in cytokinin levels in the shoot. Thimann [44] has discussed evidence that a considerable quantity of the cytokinin in axillary buds is produced therein, rather than being supplied from roots. Few studies have attempted to investigate the turnover of root-derived cytokinins in the shoot or the contribution biosynthesis in the shoot may make to the shoot cytokinin status [see references in 6]. After investigating cytokinin levels in reciprocally grafted WT and *ipt* cytokinin overproducing plants, Faiss et al. [18] have suggested that root-derived cytokinins have little effect on cytokinin content in the shoot. Along with measurement of shoot cytokinin levels, the supply [see 5, 17] and metabolism of root-derived cytokinins in WT and *ramosus* shoots is currently being investigated. In the meantime, we have not made the assumption that *rms1*, *rms3* and *rms4* shoots are deficient in cytokinin.

4.2 *Evidence for a branching induced feedback signal*

Reciprocal grafting studies with *rms4* and WT seedlings have indicated that the shoot, rather than the root, controls xylem sap cytokinin concentration [6] (Figure 4). Xylem sap obtained from rootstocks of reciprocally grafted WT and *rms4* plants contained low cytokinin concentrations only if the scion genotype was *rms4*. Furthermore, WT/*rms4* plants also had xylem sap cytokinin concentrations similar to WT/WT plants (notation; scion/rootstock). Reciprocal grafts between *rms3* and WT seedlings have provided similar results (Figure 4). Mutant *rms3* scions caused the xylem sap cytokinin concentration in WT rootstocks to fall to a similar value to that of *rms3/rms3* self-grafts. Similarly, the xylem sap cytokinin concentration from *rms3* rootstocks grafted with WT scions increased substantially compared with *rms3/rms3* self-grafts. The ability of the scion to alter the concentration of cytokinin in xylem sap obtained from roots indicates presence of a graft-transmissible signal that moves in the direction of shoot-to-root and regulates cytokinin export from the roots [6].

There are at least two likely explanations for the feedback down-regulation of cytokinin export from the roots by *rms3* and *rms4* shoots: (i) direct action; e.g., *rms3* and *rms4* may confer a hypersensitive response to cytokinin and (ii) indirect action; branching induction or bud release may cause the feedback [6]. An alternative explanation, not con-

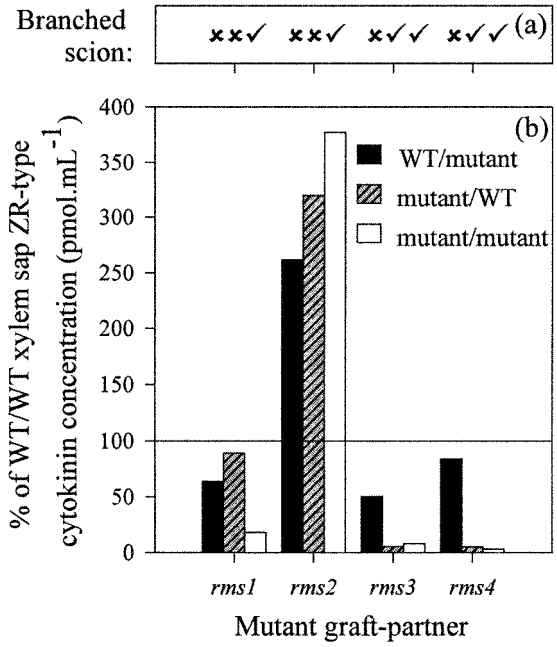

Figure 4. Branching phenotype (a) and ZR-type cytokinin concentration in the xylem sap from the roots (b) of reciprocally grafted WT (Parvus) and *rms1-1* plants and WT (Torsdag) and *rms2-1*, *rms3-2* and *rms4-1* plants. All plants were grafted epicotyl to epicotyl on day 7. Parvus and *rms1-1* plants had 11–15 leaves expanded and Torsdag, *rms2-1*, *rms3-2* and *rms4-1* plants had 17 to 20 leaves expanded at harvest. Xylem sap was harvested using a syringe-suction method and cytokinins from individual plants analysed by ELISA as described by Beveridge et al. [6]. Symbols for the branching phenotype are as described in Table 1. Data are presented as a percentage of the concentration in corresponding WT self-grafts; SEs ranged from 10–30% of the mean concentration for each graft-combination; n = 5 to 10. Photoperiod 16 h.

sidered likely at present, is that cytokinins (or their precursors) are produced mostly in the shoot, transported to roots via phloem and returned to the shoot via xylem.

Results from reciprocal grafts between WT and *rms1* seedlings indicate that an aspect of the branching process itself may be important for controlling xylem sap cytokinin concentrations. Like *rms3* and *rms4*, *rms1* plants have reduced xylem sap cytokinin concentrations compared with WT plants [9]. However, unlike *rms3* and *rms4* scions, branching is inhibited in *rms1* scions grafted to WT rootstocks. Figure 4 shows that the inhibition of branching in *rms1* scions by grafting to WT rootstocks was accompanied by an increase in xylem sap cytokinin concentration. That is, mutant *rms1* self-grafts had a highly branched phenotype and low xylem sap cytokinin concentrations whereas all plants of other graft-combinations had little or no bud release and had similar xylem sap cytokinin concentrations to that of WT/WT control plants. In this reciprocal grafting study, neither the genotype of the shoot, nor rootstock appeared to dictate the xylem sap cytokinin concentration.

The possibility that shoot branching somehow reduces xylem sap cytokinin content can be further tested in intact WT plants by inducing branching through application of exogenous cytokinin directly to the axillary buds. In the experiment presented in Figure 5, various concentrations of a synthetic cytokinin, benzyl adenine (BA), were applied to numerous nodes of WT plants. Although the differences obtained were small, plants with about two axillary buds $\geq$ 1 cm had lower xylem sap cytokinin concentrations than plants with less than one axillary bud $\geq$ 1 cm. These results are consistent with the hypothesis that the induction of axillary bud outgrowth or the presence of axillary shoots leads to the feedback down-regulation of cytokinin export from the roots.

4.3 *Do* rms2 *plants lack the feedback signal and is this signal auxin?*

In contrast with other *ramosus* mutants, *rms2* scions and rootstocks may cause slightly elevated xylem sap cytokinin concentrations in comparison with WT self-grafts [9] (Figure 4). If, as suggested above, bud outgrowth in pea leads to reduced cytokinin export from the roots, then *rms2* plants clearly lack this regulation. More direct evidence that *rms2* plants may lack the ability to down-regulate cytokinin export from the roots comes from the double mutant *rms1 rms2* [9, 38]. Unlike *rms1* plants, double mutant *rms1 rms2* plants do not have a reduced cytokinin concentration in the xylem sap indicating that *rms2* blocks the effect of *rms1* on xylem sap cytokinin concentration [9].

Although mutant *rms2* plants may lack the ability to down-regulate cytokinin export, it is yet to be established whether the slightly increased xylem sap cytokinin concentration contributes to bud outgrowth in *rms2* shoots. Bud outgrowth is greatly reduced in *rms2* scions grafted to WT rootstocks with little reduction in the xylem sap cytokinin concentration (Figure 4). Faiss et al. [18] have not found a correlation between cytokinin concentrations in the roots and bud outgrowth in the shoot. Furthermore, it is yet to be determined if the increased xylem sap cytokinin concentration in *rms2* plants equates to an increase in cytokinin delivery to the shoot [see 17]. The putative

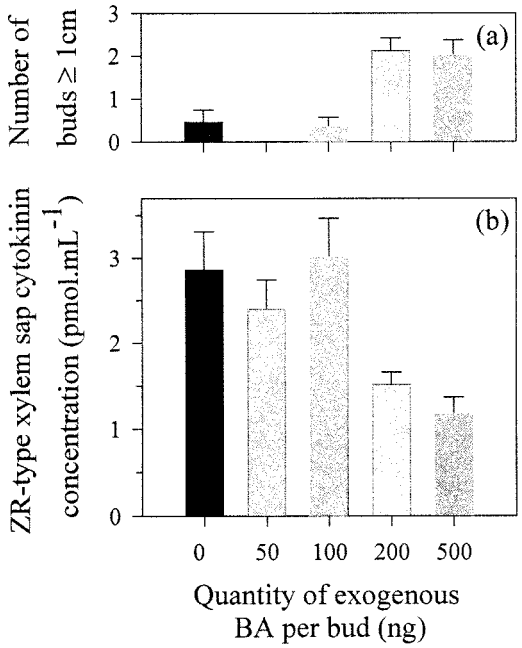

Figure 5. Branching phenotype (a) and ZR-type cytokinin concentration in the xylem sap from the roots (b) of WT (Torsdag) plants induced to branch by applying various concentrations of BA to axillary buds from nodes 8 to 11. The BA treatment (10 μl of 50% EtOH, 8% polyethylene glycol in water supplied directly to the leaf axil) did not cause substantial outgrowth of more than one bud at a node. BA was supplied 7 days before harvest at which time the plants had about 11 leaves expanded. Xylem sap was harvested using a syringe-suction method and cytokinins from individual plants analysed by ELISA as described by Beveridge et al. [6]. Data are presented as mean + SE; n = 5 to 9. Photoperiod 16 h. These results have been replicated in a second experiment (data not shown).

action of *Rms2* is discussed further later in relation to the interaction of *Rms2* with *Rms1*.

If auxin were to act as the feedback signal that regulates cytokinin export from roots of intact plants, as it may do in auxin-treated decapitated plants [4, 24], then high auxin levels would reduce cytokinin export from roots and low auxin levels would cause an increase. However, the feedback signal hypothesised to be controlled by the *Rms2* gene (Figure 2) is probably not indole-3-acetic acid (IAA) because, as discussed below, *rms2* plants have high (rather than low) endogenous IAA levels [7, 9] and efficient basipetal IAA transport in the shoot [10].

5. Auxin studies with *ramosus* mutant plants

Endogenous auxin levels have been obtained from *ramosus* mutant and WT tissues [7–9] to investigate possible differences in auxin supply from the apical bud and young leaves to nodes below bearing buds of different ages. Exogenous auxin studies using low to extremely high quantities of auxin have addressed the possibility that auxin deficiency could be responsible for the mutant phenotype or that the mutants have an altered auxin response [10] (Figure 6). Similarly, auxin transport experiments have been conducted [10] (Table 2). Results from auxin studies are summarised in Table 1.

5.1 *Auxin level is not reduced in* ramosus *mutant shoots*

Endogenous IAA levels obtained from tissues surrounding and including axillary buds at three developmental stages (within the apical bud and young nodal segments) have provided no evidence to indicate that any of the *ramosus* mutants are auxin-deficient. Rather, *rms1*, *rms2* and *rms3* shoot tissues actually contain elevated IAA levels in comparison with WT plants [7–9]. The up to 5-fold increase reported for *rms2* plants may be considered as substantial because, for example, most aspects of the pleiotropic phenotype of the *superroot* (*sur1*) mutant of *Arabidopsis* [13] have been attributed to a similar increase in free IAA and IAA-conjugates. Unfortunately, levels of IAA-conjugates have not been reported for *ramosus* mutant plants.

The elevated IAA level in shoot tissues of *rms1*, *rms3* and particularly *rms2* plants has been attributed to an involvement of auxin in the inhibition of branching [7, 8]. That is, perhaps plants compensate for factors inducing branching by increasing auxin levels. The possibility that an increased number of growing axillary shoots may simply cause increased IAA levels is not supported by the observed IAA level in *rms2* scions grafted to WT rootstocks. Compared with *rms2* self-grafts, bud outgrowth is greatly reduced in *rms2* shoots grafted to WT rootstocks, but auxin levels remain elevated [7]. Furthermore, the IAA level in *rms4* plants appears similar to that of WT plants [8]. Consequently, either bud outgrowth is not the cause of high auxin levels in other *ramosus* mutant plants, or *rms4* plants lack the ability to up-regulate shoot auxin levels.

Table 2. Auxin transport in intact WT (cv. Torsdag), *rms3-2* and *rms4-1* plants. Experimental methods and conditions were essentially as described by Beveridge et al. [10]. 37 pmol of [5(n)-^{3}H]-IAA (specific activity 10^{15} Bq mol^{-1}; Amersham International, UK) in 5 μL ethanol was applied to the apical bud inside the stipules of the leaf two nodes above the highest expanded leaf of plants with about 10 leaves expanded. After 18 h, plants were divided into consecutive stem segments of 1–2 cm in length. The distances of wave maxima are from the apical bud. Photoperiod 18 h. Data are means ± SE. n = 4 or 5

	Genotype		
	WT	*rms3-2*	*rms4-1*
Quantity of radioactivity exported from apical region per plant (pmol equivalents)	2.29 ± 0.42	4.13 ± 0.94	3.57 ± 0.47
Distance of wave maxima (cm)	7.8 ± 0.9	8.2 ± 0.4	8.5 ± 0.4

5.2 *Auxin transport is not reduced in* ramosus *mutant shoots*

The basipetal transport of radio-labelled IAA (^{3}H-IAA) supplied to the apical bud of intact plants was at least as far over 18 to 19 h in *rms1*, *rms2* [10], *rms3* and *rms4* plants (Table 2) as in WT plants. Chromatographic separation of the radioactivity transported from the apical bud of all genotypes indicated that greater than 90% of the transported radio-label was free ^{3}H-IAA ([10] and data not shown). Consequently, branching in *rms1* through *rms4* plants does not appear to be due to a reduction in auxin transport.

5.3 ramosus *mutants* rms1 *through to* rms4 *have a reduced auxin response*

Measurement of the response of axillary buds of intact *ramosus* mutant plants to exogenous auxin is problematic because of the difficulty of obtaining a WT branching control. Decapitation may be used as a means to promote branching in WT plants as it causes a rapid axillary bud outgrowth response that is essential for plant survival. Although this abrupt bud response is ideal for time-course studies, decapitation may override the more subtle regulation of plant architecture that occurs in accordance with developmental or environmental cues [28]. It is unclear what effects decapitation may have other than through removal of an auxin supply [see e.g., 20, 26, 32, 36, 40]. In a number of species, including pea, exogenous auxin applied to the decapitated stump cannot always inhibit bud outgrowth to the same extent as the intact apical bud and/or causes a number of effects, such as leaf epinasty or stem swelling, not normally observed in intact plants [e.g., 10, 15] (data not shown). Although this lack of complete inhibition could be explained by effect(s) of the exogenous auxin supply method, it could also be explained by the possibility that other signals or factors are also affected by removal of the apical bud.

Decapitation studies with *ramosus* plants may enable valuable comparisons of branching control in intact and decapitated plants. To account for differences in lateral bud size between *ramosus* and Torsdag plants, primary lateral buds can be removed from plants of both genotypes to leave secondary buds that remain inhibited if the shoot tip is not removed [10]. Decapitation then causes release of these secondary buds in mutant and WT shoots [10] (Figure 6). Intriguingly, even though *Ramosus* genes differ greatly in site of action (Table 1), mutant *rms1*, *rms2* [10], *rms3* and *rms4* plants (Figure 6) all have a reduced response to exogenous auxin applied to the decapitated stump, compared with WT plants. Total lateral length was reduced 4-fold in decapitated WT plants with 0.5 g/L IAA (about 10 μg) compared with the 0 g/L treatment (significant at $P < 0.001$; Figure 6). In contrast, bud outgrowth remained vigorous in mutants *rms1* through *rms4* even replenished daily with 20 g/L IAA (about 400 μg) [10] (Figure 6 and data not shown). It seems unlikely, though not impossible, that auxin levels at or near the bud are unaffected in all mutant plants by this largest auxin application regime [10].

5.4 *Auxin interacts with signals regulated by* Rms1 *and* Rms2

The reduced IAA-response of decapitated *rms1* and *rms2* plants is probably not caused by a defective auxin receptor or auxin signal cascade, but rather by altered levels of the graft-transmissible substances

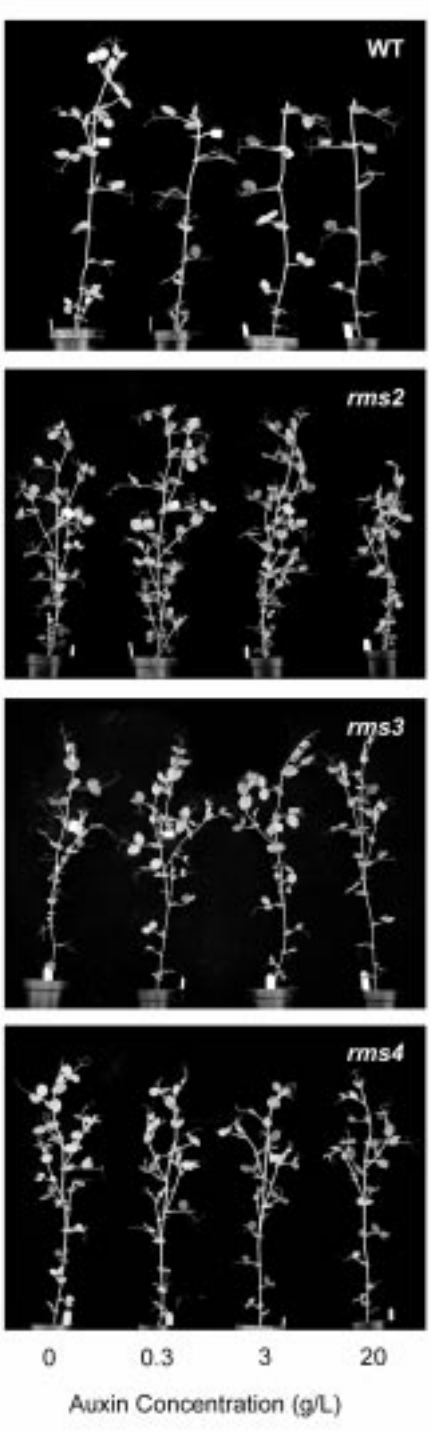

Figure 6. Response of secondary buds to exogenous IAA supplied to the decapitated stump of WT (Torsdag), *rms2-1*, *rms3-2* and *rms4-1* plants after removal of primary buds as described by Beveridge et al. [10]. Approximately 20 μl of the auxin/lanolin mix was replenished daily and representative plants were photographed after 10 days. n = 5 to 10. Photoperiod 18 h.

affected by genes at these loci. Through grafting experiments and auxin response measurements with decapitated grafted plants, Beveridge et al. [10] have recently shown that grafting *rms1* and *rms2* scions to WT rootstocks restores the ability of decapitated shoots to respond to exogenous IAA. Consequently, auxin may require the signals controlled by *Rms1* and *Rms2* to inhibit branching in decapitated plants. Alternatively, *Rms1* and/or *Rms2* may down-regulate levels of a branching promoter that is antagonistic to auxin action. It is possible that *rms3* and *rms4* are affected more directly in auxin reception or signal transduction. Equally possible however, is that these mutations affect action of the signals regulated by *Rms1* and/or *Rms2* and are therefore less able than WT to respond to auxin.

6. *Rms1* may affect the level or transport of a novel branching signal

Three pieces of evidence described above lead to the conclusion that the *Rms1* gene probably controls the level or transport of a novel signal. First, branching is inhibited in *rms1* scions by grafting to WT rootstocks [9]. Second, the cytokinin concentration in xylem sap from *rms1* plants is significantly reduced, rather than elevated, compared with WT xylem sap [9]. Third, *rms1* shoots are not auxin deficient [9] and do not have reduced basipetal auxin transport [10]. Additional support comes from the xylem sap cytokinin concentration in WT rootstocks grafted with *rms1* scions (Figure 4). Grafting *rms1* scions to WT rootstocks restores the branching phenotype to near-WT and increases xylem sap cytokinin concentrations rather than causing a further depletion (Figure 4). Following scrutiny of the interaction between *Rms1* and *Rms2* [9; discussed below], the signal controlled by *Rms1* is hypothesised to move in the direction root-to-shoot or acropetally in the shoot.

7. Interaction between *Rms1* and *Rms2*

The similarities between *rms1* and *rms2* are that both affect the level or transport of graft-transmissible signals and both cause elevated shoot auxin levels [9]. However, as mentioned above, the double mutant, *rms1 rms2* has a transgressive or additive phenotype indicating that *Rms1* and *Rms2* may control different processes [9, 38]. The possibility that one or both

mutations are leaky reduces confidence in this conclusion [9].

The hypothesis that *Rms1* and *Rms2* control interdependent pathways or processes with different end products is supported by the following argument [9]. It may also be inferred from this argument that *Rms1* and *Rms2* control signals that move in opposite directions along the plant axis (Figure 2). Grafting studies show that bud outgrowth is somewhat reduced in the graft-combination of *rms1* scions grafted to *rms2* rootstocks but not in the reciprocal combination of *rms2* scions grafted to *rms1* rootstocks. Considering the shoot phenotypes of these grafted plants, *Rms1* may control a signal that moves in the direction of root-to-shoot and/or *Rms2* a signal that moves in the opposite direction, shoot-to-root (Figure 2). Mutant *rms1* and *rms2* plants differ substantially in terms of xylem sap cytokinin concentration, being greatly reduced and slightly elevated, respectively, in comparison with WT plants. The higher than WT cytokinin concentration in the xylem sap of the *rms1 rms2* double mutant indicates that *rms2* overrides the effect of *rms1* on xylem sap cytokinin concentration. This is also consistent with the aforementioned hypothesis that *rms2* shoots lack the feedback signal required to down-regulate cytokinin delivery from the roots. These studies also indicate that *Rms2* may, directly or indirectly, regulate the root-to-shoot signal controlled by *Rms1*.

8. Conclusion

Commencing with a branching mutant and posing the question 'are hormones involved?' is a very different situation to altering hormone level or response and posing the question 'is branching affected?'. Studies with the *ramosus* series have indicated roles for two apparently novel graft-transmissible signals and have provided little direct evidence that auxin and cytokinin regulate branching in intact plants.

The hypothesis that best accounts for the known actions of the *ramosus* mutations is presented in Figure 2. The purpose of the model is to provide a unified hypothesis of the current data and hence forms a basis for discussion and hypothesis testing. In this model, genes *Rms3* and *Rms4* may control hormone perception, signal transduction or other processes in the system that occur mostly in the shoot. Genes *Rms1* and *Rms2* act in rootstock and shoot. *Rms1* controls the level or transport of a signal other than either cytokinin or an auxin precursor, that moves in the direction root-to-shoot. *Rms2* may control the level or transport of a feedback signal other than auxin that moves in direction shoot-to-root. This feedback signal may down-regulate cytokinin export from the roots and regulate *Rms1*. At least in decapitated plants, the action of exogenous auxin appears to be regulated by long-distance signals controlled by *Rms1* and *Rms2* [10]. The model therefore supports the notion proposed by Snow [37] some decades ago that auxin may act indirectly through another signal [see also 11, 19, 33].

The roles of cytokinin and auxin as promoter and inhibitor respectively, are included in the model (Figure 2) based largely on data from studies that have attempted to correlate bud outgrowth with the levels of, or response to, these hormones [e.g., 4, 12, 21, 24, 34]. The strongest genetic evidence *ramosus* mutants have provided in support of these roles is the indication that feedback mechanisms appear to be in place that down-regulate, or up-regulate endogenous xylem sap cytokinin concentrations and shoot auxin levels, respectively.

The relatively non-pleiotropic phenotype of *ramosus* mutant plants indicates that the long-distance signals regulated by *Rms1* and *Rms2* may be quite specific to the branching process. Alternatively, if these signals are not specific to branching, then *Rms1* and *Rms2* may affect their compartmentalisation such that only bud outgrowth is regulated by these genes. Either way, once *Ramosus* or related genes have been sequenced, they may prove very useful for agronomic applications requiring the manipulation of shoot architecture. From a less applied point of view, the *Ramosus* genes are intriguing as they may lead us to the identification of novel phytohormone-like signals.

9. Future directions

Reverse mutageneses of *rms3* and *rms4* mutants have defined mutations that suppress branching and hence may represent loci of branching promotion genes (C Rameau, per. comm.). Together with the *ramosus* series, mutants obtained through this approach, should allow us to investigate the genetic control of both the promotion and inhibition of bud outgrowth.

Future studies will expand on auxin responses in intact plants rather than rely solely on decapitated plants. It is important to determine if the processes underlying bud outgrowth in intact and decapitated plants are mutually exclusive or not [28]. For example,

are signals such as auxin and cytokinin more important for regulating the response of axillary buds to decapitation than for the control of bud outgrowth in intact plants? A further challenge remains in determining and interpreting any small or very localised differences in hormone concentrations in WT and mutant axillary buds and in investigating hormone reception and signal transduction.

Identification of novel signals and pathways regulating branching in pea may be achieved through a combination of bioassays and biochemistry, and/or through determining gene functions at the molecular level. The advent of microarray technologies [45], the development of pea BAC libraries (Dr. Khalid Meksem, pers. comm.) and the sequencing of other plant genes and genomes [e.g., 46] will facilitate isolation of branching genes in pea. In order to complete the chain of regulation from long-distance signalling in the whole plant through to gene action at the molecular level, the study of plant architecture may require research approaches that take advantage of the particular features of a number of model species.

Acknowledgements

I thank Profs Ian Murfet and Peter Gresshoff, Drs Catherine Rameau, John Ross and Colin Turnbull for their helpful comments on the manuscript and/or for great discussions of branching in pea, Drs Jimmy Botella and Ian Dodd for specialist contributions, Dr John Ross and Greg Symons, in particular, for help with the auxin transport experiment presented in Table 2, Prof Emile Miginiac, Dr Bruno Sotta and M. Bonnet for assistance with ELISA measurements, Dr Christa Critchley for encouragement and useful comments, Suzanne Morris for further valuable contributions and the Australian Research Council and the University of Queensland for financial support. Some of the data presented was obtained at the Station de Génétique et d'Amélioration des Plantes, Institut National de la Recherche Agronomique (INRA, France), the Laboratoire de Physiologie du Développement des Plantes, Université Pierre et Marie Curie (France) or at the University of Tasmania (Australia).

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