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Branching in Pea[1]

Action of Genes *Rms3* and *Rms4*

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The nonallelic *ramosus* mutations *rms3–2* and *rms4* of pea (*Pisum sativum* L.) cause extensive release of vegetative axillary buds and lateral growth in comparison with wild-type (cv Torsdag) plants, in which axillary buds are not normally released under the conditions utilized. Grafting studies showed that the expression of the *rms4* mutation in the shoot is independent of the genotype of the rootstock. In contrast, the length of the branches at certain nodes of *rms3–2* plants was reduced by grafting to wild-type stocks, indicating that the wild-type *Rms3* gene may control the level of a mobile substance produced in the root. This substance also appears to be produced in the shoot because *Rms3* shoots did not branch when grafted to mutant *rms3–2* rootstocks. However, the end product of the *Rms3* gene appears to differ from that of the *Rms2* gene (C.A. Beveridge, J.J. Ross, and I.C. Murfet [1994] Plant Physiol 104: 953–959) because reciprocal grafts between *rms3–2* and *rms2* seedlings produced mature shoots with apical dominance similar to that of *rms3–2* and *rms2* shoots grafted to wild-type stocks. Indole-3-acetic acid levels were not reduced in apical or nodal portions of *rms4* plants and were actually elevated (up to 2-fold) in *rms3–2* plants. It is suggested that further studies with these branching mutants may enable significant progress in understanding the normal control of apical dominance and the related communication between the root and shoot.

The term apical dominance refers to the mechanism whereby a plant maintains the growth of a main shoot at the expense of lateral branches. Branching, or reduced apical dominance, results from bud release and subsequent bud growth. A number of stages may be traversed during this process, and at least in pea (*Pisum sativum* L.), each stage appears to be correlated with a unique profile of proteins in the axillary bud (Stafstrom and Sussex, 1988; Stafstrom, 1993). Therefore, it is quite probable that a number of genes act either in sequence or in parallel to control various stages of bud release and growth. Furthermore, phytohormones may influence gene action at some or all of these stages. Recent studies involving decapitation, auxin application, and cytokinin assays have indicated that a dynamic interaction may exist between the shoot and roots in terms of auxin and cytokinin levels (Bangerth, 1994; Li et al., 1995).

It is widely accepted that IAA and cytokinin, but not ethylene (Romano et al., 1993), are the primary factors that control apical dominance (Klee and Estelle, 1991; Cline, 1994). However, despite the production of plants with artificially altered endogenous cytokinin (e.g. transgenic *ipt* plants; Medford et al., 1989) or IAA levels (transgenic *iaaH*, *iaaM*, and *iaaL* plants; Romano et al., 1991; Sitbon et al., 1992), it is not understood precisely how these hormones interact to control branching. Furthermore, it has not been established whether these hormones affect both bud release and subsequent growth, and more information on the temporal and spatial variation in hormone levels is required. For example, if one accepts the theory that low endogenous IAA levels promote bud release, then it is difficult to reconcile the fact that juvenile transgenic 35S-*iaaL* tobacco plants with up to 19-fold less IAA than comparable WT plants do not release buds at a lower node than WT plants (Romano et al., 1991; C.P. Romano, personal communication). In fact, this latter observation is consistent with the hypothesis of Sachs and Thimann (1967) that cytokinins are involved in the release of buds from inhibition, whereas auxin promotes only the subsequent elongation of buds. That IAA is involved in the control of apical dominance is rarely disputed, but its particular role has yet to be precisely determined.

If it is accepted that changes in hormone levels in certain transgenic plants are correlated with the timing of events involved in the process of branching, the next step is to determine if changes in hormone level or tissue sensitivity occur under normal conditions of branching (Cline, 1994). Therefore, studies with branching mutants have a number of advantages because they necessarily involve alterations in the normal control mechanisms of the intact plant. Consequently, the *amp1* mutant of Arabidopsis, which has elevated cytokinin levels at the rosette stage (Chaudhury et al., 1993), is very valuable. This mutant differentiates a greater number of leaves than the WT (Columbia) plants and an increased number of elongated flowering shoots from the multiplied rosette. It is hoped that correlations of the endogenous cytokinin levels with the timing of bud release (or shoot multiplication), bud growth, and floral evocation in this mutant will provide a valuable insight into the role of cytokinin in the branching process. How-

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Abbreviations: TLL, total lateral length; WT, wild-type.

ever, new mutants are still required and may provide the necessary insight into the possibility that novel substances (see Tamas et al., 1992) or unpredicted mechanisms are involved in the control of apical dominance in some species.

The garden pea is an excellent plant for studies of apical dominance. Several nonallelic mutants with increased branching are available and environmental conditions can be chosen in which lateral bud release occurs in the mutant plants but is completely, or almost completely, inhibited in WT plants (Arumingtyas et al., 1992). Furthermore, the architecture of the pea plant is such that the main stem is easily identified and, although several axillary buds may be produced in the leaf axils, generally only one bud per axil is released at the upper nodes when branching is promoted (Arumingtyas et al., 1992). We showed previously that the *Rms2* gene appears to maintain apical dominance in pea by controlling the production of a graft-transmissible substance that affects branching (Beveridge et al., 1994). In addition to increased branching, the *rms2* mutant was also characterized by increased IAA levels and shorter internodes. In this study, we have investigated the action of two further branching genes in pea, *Rms3* and *Rms4* (Arumingtyas et al., 1992), using reciprocal grafts between mutant and WT seedlings and by analysis of endogenous IAA levels. The possibility that *Rms3* or *Rms4* may act on the same system as the *Rms2* gene was investigated by reciprocal grafts among the three mutant lines.

MATERIALS AND METHODS

Plant Materials

The mutant lines used in this study were all obtained from the WT pea (*Pisum sativum* L.) cv Torsdag following treatment with ethyl methanesulfonate and were kindly provided by Dr. K.K. Sidorova (Institute of Cytology and Genetics, Novosibirsk, Russia). All three mutants are recessive, nonallelic, and characterized by a fairly similar pattern of increased branching (Arumingtyas et al., 1992). Line K524 carries mutant allele *rms2*, line K564 carries allele *rms3–2*, and line K164 carries allele *rms4*. Torsdag carries the WT dominant alleles *Rms2*, *Rms3*, and *Rms4*.

Methods

All plants were grown in a heated glasshouse with the natural photoperiod extended to 18 h with a 1:1 mixture of white fluorescent (40 W) and incandescent (100 W) lights. Seeds were planted in 14-cm slim-line pots containing a 1:1 (v/v) mixture of vermiculite and 10-mm dolerite chips, topped with 4 cm of pasteurized peat/sand potting mix. Nutrient solution (Aquasol Hortico Ltd., Melbourne, Australia; or Total Growth Nutrient, R&D Aquaponics, Sydney, Australia) was supplied to the plants once a week. Nodes were numbered acropetally from the first scale leaf as node 1. Lateral lengths were measured from the base of the lateral (in the leaf axil) to the apex of the lateral shoot. Basal laterals were removed from all nongrafted plants (usually at d 17) to encourage uniform growth of the main stem among all plants. The TLL was the sum of the lateral lengths at each node along the main stem.

Grafting Technique

Grafts were performed prior to any macroscopic sign of bud release as described by Beveridge et al. (1994). The seedlings were 6 d old at the time of grafting, and scions (shoot) and stocks (roots and cotyledons) were joined at the epicotyl by a wedge connection. Lateral shoots were excised from the cotyledonary node because they weaken the growth of the scion. Only vigorous plants (usually more than 90% of grafted plants) were included in the analysis.

IAA Extraction, Purification, and GC-MS Analysis

The endogenous IAA was quantified as described by Beveridge et al. (1994). In brief, the freshly harvested tissue was placed directly into methanol/butylated hydroxytoluene at −20°C. Deuterated IAA (indole-2,4,5,6,7-$^2H_5$-IAA; synthesized by Merck and Co., Rahway, NJ) was added as the internal standard. The purification procedure included solvent partitioning (diethyl ether) and a Sep-Pak (Waters Associates, Melbourne, Australia) C_{18} cartridge step. Prior to GC-MS-selected ion monitoring analysis, the samples were methylated and trimethylsilylated. The endogenous IAA levels were calculated by comparison with the internal standard, using the isotope dilution method described by Cohen et al. (1986).

RESULTS

Mutant Phenotypes

The patterns of branching along the stem of the *rms2*, *rms3–2*, and *rms4* mutant plants are fairly similar (Fig. 1). Under the 18-h photoperiod, all mutant plants tended to produce one or more vigorous basal laterals (from nodes 1–3) and aerial laterals above node 7, with the most vigorous of these usually above node 10 (Fig. 1; Arumingtyas et al., 1992). The total lateral length of plants at d 17 therefore consisted mainly of lateral outgrowth at nodes 1 to 3 (because there were no more than seven or eight leaves expanded on 17-d-old plants; Table I). At d 17, the basal outgrowth of *rms2* plants was over twice the length of that of *rms3–2* and *rms4* plants. The excision of basal laterals at this time promotes the outgrowth of aerial lateral buds on mutant plants and causes the plants of all genotypes to be essentially devoid of basal laterals at later stages (Fig. 1). Excising the basal laterals from *rms2* plants at d 17 did not influence the total lateral length measured at d 64 (Fig. 1; the mean TLL values for intact and basal-lateral-excised *rms2* plants were 688 ± 111 and 688 ± 196 mm, respectively). In contrast to the basal lateral growth, the lateral lengths above node 7 were greater in *rms3–2* and *rms4* plants than in *rms2* plants (Table I; Fig. 1).

The internodes of *rms2*, *rms3–2*, and *rms4* plants tended to be shorter than those of the WT plants (Fig. 2; Arumingtyas et al., 1992). The largest differences between WT plants and *rms3–2* and *rms4* plants were recorded for the stem intervals between nodes 6 and 9 and between nodes 9

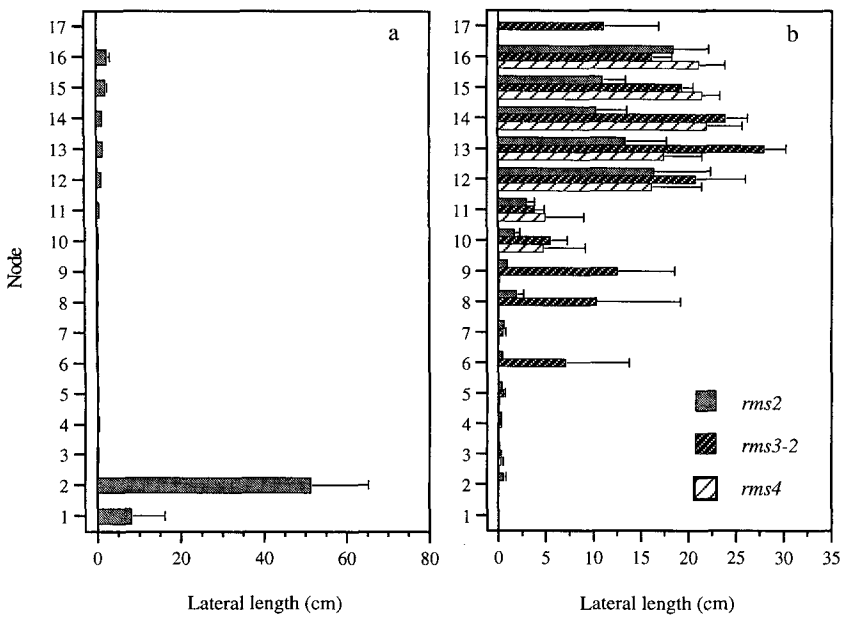

Figure 1. Lateral lengths at each node of 64-d-old intact *rms2* plants (a) and *rms2*, *rms3–2*, and *rms4* mutant plants (b) following lateral removal from nodes 1, 2, and 3 at d 17. Data shown are means + SE; n = 6 to 9.

and 12. The reduction in internode length above node 6 in mutant plants, compared with WT plants, was less extreme for *rms3–2* and *rms4* plants than for *rms2* plants (Fig. 2). The small differences between the flowering node of the WT and various mutant plants (Table I) were not observed in all experiments (data not shown).

Unlike the stem width of *rms2* plants (Arumingtyas et al., 1992; Beveridge et al., 1994), the stem width of *rms3–2* and *rms4* plants was not significantly less than that of WT plants (Table II). Although there were no significant differences in the leaflet breadths of mutant plants, compared with those of WT plants, the leaflet length of *rms2* and *rms4* plants was 22 and 17%, respectively, less than that of WT plants (Table II).

IAA Levels

Endogenous IAA levels in *rms3–2*, *rms4*, and WT plants were determined at the time of aerial lateral bud release in mutant plants for three ontogenetically different portions: the apical portion (above the oldest unexpanded leaf), the node at the uppermost expanded leaf, and the node below it (Fig. 3). The IAA level was typically elevated in the nodal portions of *rms3–2* shoots. Although this accumulation of IAA was less than 2-fold, it was observed in all comparisons of *rms3–2* and WT nodal segments. However, little or no accumulation of IAA was observed in the apical portion of *rms3–2* plants in comparison with the level in WT apical portions. In contrast, the level of IAA in *rms4* plants did not differ significantly from that found in WT plants (Fig. 3). Similar results were obtained when the IAA levels in mutant and WT plants were compared on a per portion basis (rather than a per gram basis as in Fig. 3).

Site of Action

The *Rms3* gene appears to control the level of a graft-transmissible substance, since branching in *rms3–2* mutant scions was partly inhibited by grafting to WT stocks (Fig. 4). However, in contrast with *rms2* scions grafted to WT stocks, the inhibition of branching in *rms3–2* scions was confined to the region below node 10. In the node 6 to 10 region of the stem, the aggregate lateral length of *rms3–2*/WT plants (19.2 ± 6.1 cm) was less than one-fourth that of *rms3–2*/*rms3–2* plants (82.0 ± 12.0 cm). Similar results were obtained when *rms3–2* scions were grafted to other WT stocks (cv's Parvus and Weitor; data not shown). Furthermore, in contrast to *rms2* scions grafted to WT stocks,

Table I. *Mean ± SE for the total main stem length (TL), number of leaves expanded on the main stem (LE), and TLL of mutant and WT plants at d 17 and 64*

FI and n refer to the node of flower initiation and the number of plants, respectively. Basal laterals were removed, after scoring, on d 17.

Genotype	d 17			d 64			FI	n
	TL	TLL	LE	TL	TLL	LE		
	cm		*node*	*cm*		*node*	*node*	
WT	27.3 ± 0.4[a]	0.0 ± 0.0[a]	7.8 ± 0.1[a]	135.1 ± 1.2[a]	0.6 ± 0.1[a]	19.9 ± 0.1[a]	16.3 ± 0.2[a]	9
rms2	20.1 ± 0.6[b]	7.8 ± 1.0[b]	7.3 ± 0.6[a]	107.3 ± 4.6[b]	68.8 ± 19.6[b]	21.9 ± 0.4[b]	16.6 ± 0.3[a]	7
rms3–2	26.5 ± 0.9[a]	2.1 ± 0.4[c]	8.2 ± 0.1[a]	126.8 ± 1.8[c]	154.1 ± 11.7[c]	22.2 ± 0.2[b]	17.5 ± 0.2[b]	6
rms4	26.3 ± 0.7[a]	2.9 ± 1.2[c]	8.0 ± 0.1[a]	130.3 ± 2.1[a,c]	105.8 ± 6.1[d]	22.0 ± 0.2[b]	16.9 ± 0.2[a,b]	8

[a,b,c,d] Values within a column that have the same letter are not significantly different at the P = 0.05 level.

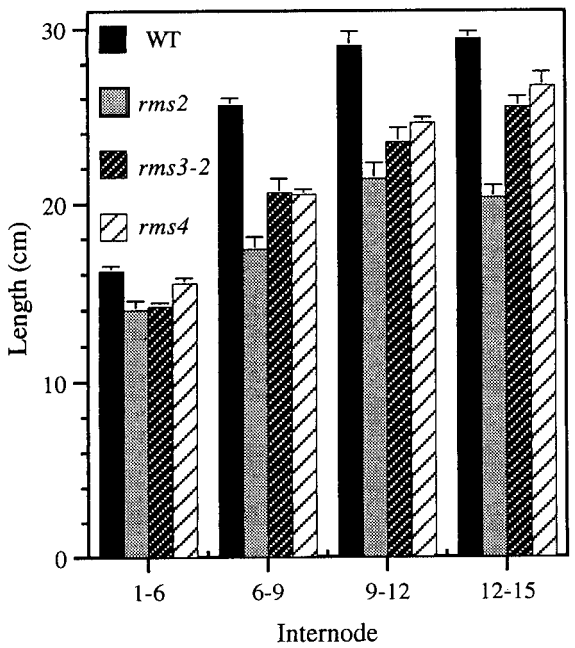

Figure 2. Stem lengths between nodes 1 and 6, 6 and 9, 9 and 12, and 12 and 15 of the main stem of 64-d-old *rms2*, *rms3–2*, *rms4*, and WT plants. The basal laterals were removed on d 17. Data shown are means + SE; n = 6 to 9.

the inhibition of branching in *rms3–2* scions was due largely to the inhibition of lateral bud growth rather than the inhibition of bud release (Table III). As in the case of the grafts of *rms2* stocks with WT scions (Beveridge et al., 1994), *rms3–2* stocks did not promote branching in WT scions (Fig. 4), indicating that the *Rms3* gene can contribute to the inhibition of lateral branching via action in either the scion or the stock.

In contrast, the action of the *Rms4* gene may be entirely confined to the shoot. Branching in *rms4* scions was not inhibited by grafting to WT stocks, and *rms4* stocks did not promote lateral bud release in WT scions (Table III). The *rms4*/*rms4* and *rms4*/WT plants did not differ significantly in either mean TLL or mean lateral length at almost any individual node (Table III; Fig. 4). Furthermore, *rms4* stocks

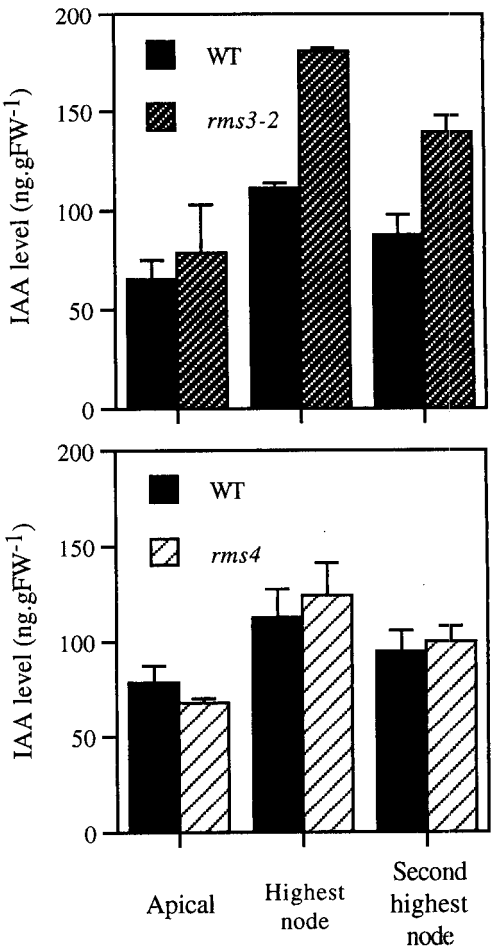

Figure 3. IAA level in various parts of *rms3–2* (top), *rms4* (bottom), and WT shoots (top and bottom). Portions harvested were the apical portions, which are those portions above the oldest unexpanded leaf; the node at the highest expanded leaf (highest node); and the node below the highest expanded leaf (second-highest node). The nodal segments consisted of a 1-cm portion of the petiole and stem on each side of the node. Branches were removed from nodes 1 to 3 at an early stage to ensure vigorous growth of the main stem. The plants had about 11 expanded leaves at the time of harvest. Data shown are means + SE for two samples, each from >10 plants. FW, Fresh weight.

Table II. *Stem width and leaf length and breadth of mutant and WT plants*

Stem width was measured at a point on the main stem approximately 5 mm above the node of flower initiation and with an axis perpendicular to the direction of the petiole at that node. The leaf measurements were recorded as maximum breadth or length of a single leaflet from the pair closest to the stem on the leaf at the node of flower initiation. Data are for means ± SE; n refers to the number of plants. All measurements are in millimeters.

Genotype	Stem Width	Leaflet Length	Leaflet Breadth	n
WT	3.52 ± 0.05[a]	43.5 ± 0.9[a]	26.0 ± 0.8[a,b]	9
rms2	3.16 ± 0.10[b]	34.1 ± 1.4[b]	23.7 ± 0.8[a]	7
rms3–2	3.52 ± 0.06[a]	41.6 ± 0.8[a]	27.4 ± 0.4[b]	6
rms4	3.51 ± 0.07[a]	36.0 ± 0.8[b]	24.9 ± 0.7[a]	8

[a,b] Values within a column that have the same letter are not significantly different at the P = 0.05 level.

were not able to promote branching in any other mutant scion, and *rms2* and *rms3–2* stocks were unable to significantly alter the TLL of *rms4* scions (Table III).

Mutant *rms2* and *rms4* stocks were perhaps even more effective than WT stocks at reducing branching in *rms3–2* scions ($P < 0.05$; Table III; Fig. 4). However, this effect was again confined to the middle and lower nodes and did not occur at the upper nodes (Fig. 4). In the reciprocal grafts, *rms3–2* and *rms4* stocks inhibited branching in *rms2* scions to an extent similar to the almost complete inhibition caused by WT stocks.

As in *rms2* scions (Table III; Beveridge et al., 1994), the reduction in branching in *rms3–2* scions when grafted to other mutant or WT stocks did not result in a proportional increase in main stem length (Table III). For example, al-

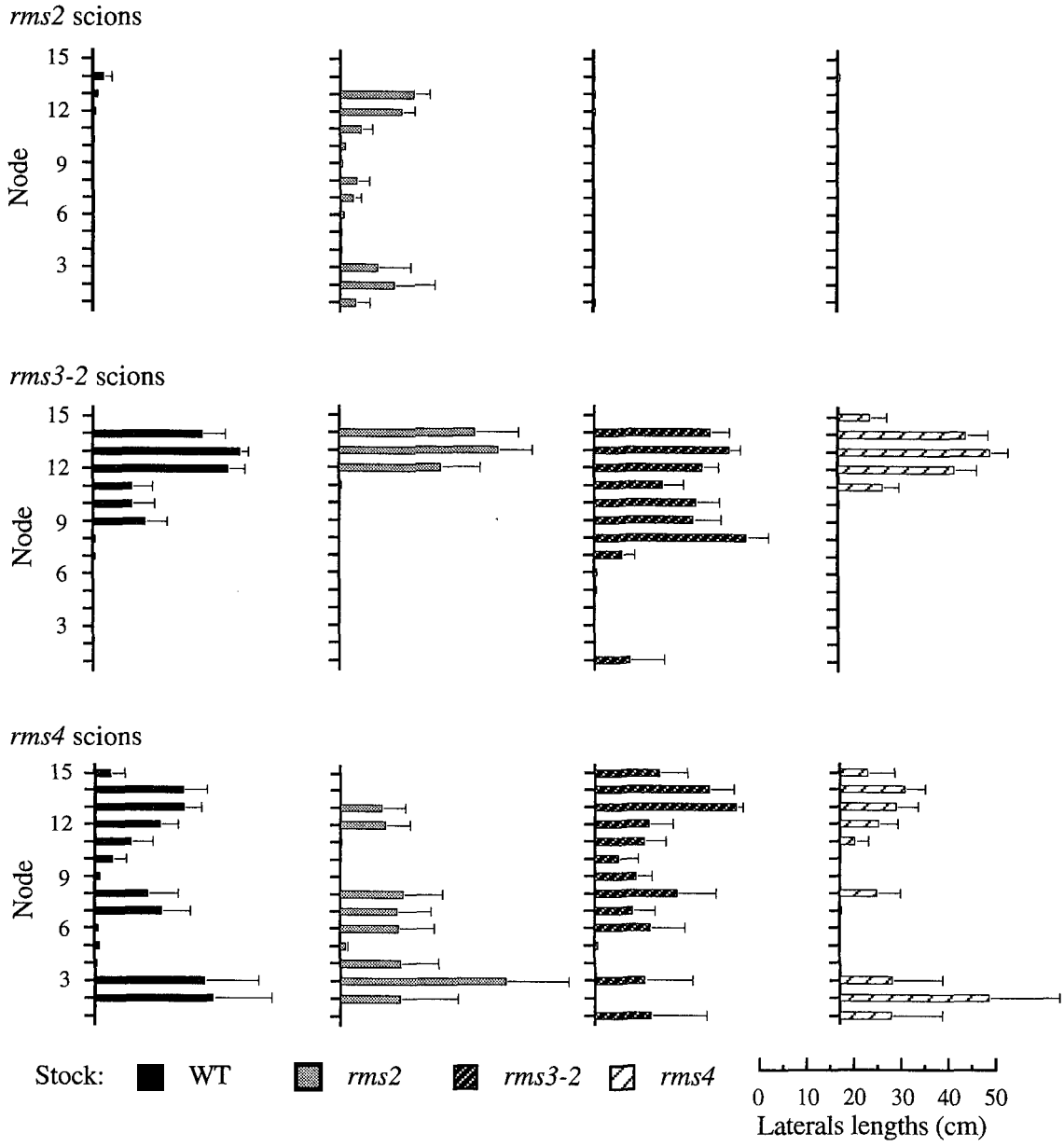

Figure 4. Lateral lengths at consecutive nodes of *rms2* (top), *rms3–2* (middle), and *rms4* (bottom) scions 46 d after grafting with WT, *rms2*, *rms3–2*, and *rms4* stocks (from left to right). Data for WT scions are not presented because WT scions did not produce significant lateral branches. $n = 6$ to 10, except for the *rms2/rms4* graft-combination, where the number of vigorous scions was only 3. Data presented are means + SE, and lengths correspond with the scale provided.

though WT stocks caused a 42% drop in the total lateral length of *rms3–2* scions, they caused only a small (but significant; $P < 0.05$) increase in the main stem length (Table III). Furthermore, although *rms2* stocks caused a 3-fold drop in the TLL for *rms3–2* scions, they did not increase the main stem length of *rms3–2* scions (Table III). In fact, the main stem length of scions of all genotypes were shortest when grafted to *rms2* stocks.

DISCUSSION

Our results (Table III; Fig. 4) indicate that the *rms3–2* mutation affects the level of a graft-transmissible substance that inhibits branching. A similar result was reported previously for the *rms2* mutation (Beveridge et al., 1994). However, certain observations indicate that two separate hormone-like substances may be involved. If the *Rms2* and *Rms3* genes acted on the same biochemical pathway, apical dominance should not have been restored in one or the other of the graft combinations *rms2/rms3–2* or *rms3–2/rms2*. However, branching was inhibited in both graft combinations, compared with the mutant self-grafts, indicating that the two genes may affect separate biosynthetic pathways.

There are significant phenotypic differences between *rms3–2* and *rms2* plants. Branching is more vigorous in

Table III. *TLL and total main stem length (TL) of 46-d-old plants from reciprocal grafts of WT (Torsdag), rms2, rms3–2, and rms4 seedlings*

The plants were the same as those represented in Figure 4. Data are means ± SE; $n = 11$ or 12, except in the case of the grafts of *rms2* scions to *rms4* stocks, where $n = 3$. The *rms2/rms4* grafts lacked vigor in most cases due to the repeated lateral bud release and growth from the cotyledonary node of the *rms4* stock. All measurements are in centimeters.

Stock	Measurement	Scion			
		Torsdag	*rms2*	*rms3–2*	*rms4*
Torsdag	TLL	0.5 ± 0.5	5.2 ± 1.5	106.0 ± 16.0	139.9 ± 13.6
	TL	125.7 ± 3.0	90.2 ± 2.4	117.9 ± 2.5	105.1 ± 2.2
rms2	TLL	0.3 ± 0.1	69.6 ± 5.8	51.1 ± 11.7	117.2 ± 15.9
	TL	113.2 ± 2.5	88.0 ± 2.3	101.8 ± 4.7	100.4 ± 2.1
rms3–2	TLL	0.1 ± 0.1	2.3 ± 0.7	181.3 ± 13.0	157.0 ± 15.4
	TL	132.0 ± 2.1	90.8 ± 2.7	111.0 ± 1.6	106.1 ± 2.8
rms4	TLL	0.1 ± 0.1	0.2 ± 0.2	76.7 ± 12.0	108.4 ± 18.1
	TL	128.3 ± 4.0	72.8 ± 11.0	113.4 ± 3.4	111.9 ± 1.8

rms3–2 plants, whereas internode length, stem width, and leaf size are more reduced in *rms2* plants. Again, WT stocks caused an almost complete inhibition of branching in *rms2* scions, but they inhibited the lateral growth only at certain nodes of *rms3–2* plants (Fig. 4). Furthermore, when scions with the *rms3–1* allele (line K487, obtained from cv Torsdag; Arumingtyas et al., 1992) were grafted to Torsdag stocks, there was no significant inhibition of branching (R.S. Floyd and I.C. Murfet, unpublished data). The two allelic mutants *rms3–1* and *rms3–2* have similar branching phenotypes (Arumingtyas et al., 1992), and the basis for the difference in response of these two mutants to grafting is not apparent at this stage.

Branching was not inhibited in *rms4* shoots when grafted to stocks of any genotype, and *rms4* stocks did not induce branching in WT scions. Thus, there is no evidence that *Rms4* controls the synthesis of a graft-transmissible substance. This does not eliminate the possibility that *Rms4* acts in a biosynthetic pathway, since, for example, no graft-transmissible effect has been demonstrated for the pea internode length gene *Le*, which controls conversion of GA_{20} to the active GA_1 (Reid et al., 1983; Ingram et al., 1984). It is possible that *Rms4* controls reception or response to a branching factor, but at this stage the action of *Rms4* remains unclear.

The profile of endogenous IAA levels in the mutants also differs; the level of IAA appears to increase from normal levels in WT and *rms4* plants, through (up to) a 2-fold increase in *rms3–2* plants, to (up to) a 5-fold increase in *rms2* plants (Fig. 3; Beveridge et al., 1994). Therefore, it does not appear that the reduced apical dominance of any of the mutant plants is attributable to a lack of endogenous IAA, although the level of IAA in localized cells of *rms4* plants has not been determined. Nevertheless, the observation that *rms4* plants have apparently normal IAA levels supports our previous suggestions (Beveridge et al., 1994) that the elevated IAA level in *rms2* plants is not simply a consequence of bud release or growth and that the substance controlled by the *Rms2* gene has a fairly direct influence on IAA level. The same conclusion may be drawn from the observation of the elevated IAA levels in *rms3–2* plants (Fig. 3). It was suggested that the elevated IAA levels in *rms2* plants may be part of a regulatory mechanism intended to maintain apical dominance. Therefore, it is noteworthy that the second pea mutant characterized as having an altered level of a graft-transmissible substance (*rms3–2*) also has elevated IAA levels, whereas the *rms4* mutant (which appears to act independently of graft-transmissible factors) has normal IAA levels in all portions tested (Fig. 3). Indeed, it is possible that the substances controlled by the *Rms2* and *Rms3* genes are involved in the regulation of IAA level and that these regulatory processes may operate prior to, or independently of, the action of the *Rms4* gene. The *axr1* mutant of Arabidopsis also has increased branching (Lincoln et al., 1990) and elevated IAA levels (Romano et al., 1995), but whether graft-transmissible factors are involved in the increased branching and reduced IAA response of these plants is not known.

Often it is difficult to distinguish between cause and consequence in processes like bud release. For example, some hormonal changes in the mutants may be the consequence of imminent or actual bud growth, whereas others (such as the elevated IAA level in *rms2* plants) may be a more direct consequence of the mutation. Therefore, the fact that there is an apparently wide variation in the action of the *rms2*, *rms3–2*, and *rms4* mutations may allow for interesting comparisons of hormone level and metabolism. The possibility that any of these mutations alters the level of cytokinin must be investigated. However, because it appears that genes *Rms2* and *Rms3* do not display physiological complementarity (i.e. do not appear to alter the same biosynthetic pathway), it is difficult at present to envisage a mechanism whereby mutations at both loci influence cytokinin levels per se. Studies are also underway to determine the response of mutant plants (and isolated segments) to exogenous IAA and cytokinin, to further examine the communication between the root and shoot in terms of hormone and gene action, and to characterize a fourth branching mutant (*rms1*).

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