

RESEARCH PAPER

Existing branches correlatively inhibit further branching in *Trifolium repens*: possible mechanisms

R. G. Thomas and M. J. M. Hay*

AgResearch Grasslands, Private Bag 11008, Palmerston North, New Zealand

* To whom correspondence should be addressed. E-mail: mike.hay@agresearch.co.nz

Received 21 April 2010; Revised 30 September 2010; Accepted 1 October 2010

Abstract

In *Trifolium repens* removal of any number of existing branches distal to a nodal root stimulates development of axillary buds further along the stem such that the complement of branches distal to a nodal root remains constant. This study aimed to assess possible mechanisms by which existing branches correlatively inhibit the outgrowth of axillary buds distal to them. Treatments were applied to basal branches to evaluate the roles of three postulated inhibitory mechanisms: (I) the transport of a phloem-mobile inhibitory feedback signal from branches into the main stem; (II) the polar flow of auxin from branches into the main stem acting to limit further branch development; or (III) the basal branches functioning as sinks for a net root-derived stimulatory signal (NRS). Results showed that transport of auxin, or of a non-auxin phloem-mobile signal, from basal branches did not influence regulation of correlative inhibition and were consistent with the possibility that the intra-plant distribution of NRS could be involved in the correlative inhibition of distal buds by basal branches. This study supports existing evidence that regulation of branching in *T. repens* is dominated by a root-derived stimulatory signal, initially distributed via the xylem, the characterization of which will progress the generic understanding of branching regulation.

Key words: Auxin, axillary bud outgrowth, branching promoter signal, correlative inhibition, nodal roots, stem girdling, *Trifolium repens*.

Introduction

Plants have the capacity to control both the number and position of axillary buds activated to form branches and how these branches grow. This self-regulation of axillary bud development is referred to as correlative control and includes the processes of both apical dominance and correlative inhibition. The latter includes both apical control, defined as the suppression of growth of an already growing branch imposed by the growth of a higher dominating branch or shoot (see Cline and Sadeski, 2002), and the inhibitory influence of basal branches on distally located axillary buds and branches (Beveridge *et al.*, 1996; Ongaro *et al.*, 2008; Dun *et al.*, 2009b; Ferguson and Beveridge, 2009). There is strong evidence of an important role for the basipetal movement of auxin in the polar auxin transport stream from the shoot apical bud down the primary stem in the regulation of apical dominance (Ongaro and Leyser, 2008), but its influence on correlative inhibition is more

debatable. For instance, whereas intra-plant transport of auxin was established as a causal factor in correlative inhibition in two-branched *Pisum sativum* (Morris, 1977; Li and Bangerth, 1999), it was ruled out as a factor in *Ipomoea nil* (Cline and Sadeski, 2002). Despite intensive study, the various pathways of auxin action in regulating the initiation and maintenance of axillary bud outgrowth have remained somewhat contentious, although very recently, following the identification of the inhibitory hormone, strigolactone (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008), progress has been made in understanding the role of auxin–strigolactone interactions (Brewer *et al.*, 2009; Dun *et al.*, 2009a, b; Ferguson and Beveridge, 2009; Hayward *et al.*, 2009; Crawford *et al.*, 2010; Liang *et al.*, 2010). The models presented by these authors are based on evidence suggesting that the polar flow of auxin from the shoot apical bud acts directly to down-regulate the AXR1/TIR1/cytokinin

synthesis pathway in the region of the stem adjacent to an axillary bud, while at the same time it up-regulates the MAX/RMS/DAD pathway for strigolactone synthesis. Polar flow of auxin from axillary buds into the main stem then plays a role in facilitating their continued development (Sachs, 1968; Brewer *et al.*, 2009). Brewer *et al.* (2009) suggest a direct role for auxin in stimulating strigolactone synthesis in the vascular cambial cells through which auxin is transported (Booker *et al.*, 2003, 2005; Sorefan *et al.*, 2003) so that strigolactone functions downstream of auxin in a manner that supports the classical second messenger theory of apical dominance (Snow, 1929, 1937). However, Crawford *et al.* (2010) found that strigolactone was not capable of inhibiting isolated buds and required auxin flow in the associated stem to become an effective inhibitor. There is little controversy regarding the down-regulating effect auxin has on cytokinin synthesis (Shimizu-Sato *et al.*, 2009) and the importance of cytokinin synthesis in the region of a stem local to a bud for the stimulation of its outgrowth (Nordström *et al.*, 2004; Tanaka *et al.*, 2006; Dun *et al.*, 2009b; Ferguson and Beveridge, 2009).

Comparatively recently, a further role for polar auxin transport in the regulation of axillary bud outgrowth has been described in a mechanism known as the auxin transport capacity theory (Leyser, 2005; Bennett *et al.*, 2006; Dun *et al.*, 2006; Ongaro and Leyser, 2008; Ongaro *et al.*, 2008). This theory is based on the premise that there is competition between an axillary bud and the main stem apex in their ability to export auxin into the auxin transport stream of the main stem as the latter has limited capacity to transport auxin and a bud must be able to export auxin into it in order to grow (Sachs, 1968, 1969). Ongaro *et al.* (2008) have described in more detail the earlier work on which the theory is based, including that of Li and Bangerth (1999) relating the concept to correlative inhibition. However, recently, Brewer *et al.* (2009) found for both pea and *Arabidopsis* that the main stems are capable of instantly transporting additional auxin in excess of endogenous levels and that direct application of the auxin transport inhibitor NPA (*N*-1-naphthylphthalamic acid) to the buds of strigolactone mutant pea plants fails to stop the initial outgrowth of buds, whereas outgrowth is completely prevented by the application of the synthetic strigolactone, G24. These findings indicate that auxin transport capacity is unlikely to have a strong controlling influence on the initiation of bud outgrowth but may influence the continued development of the bud. However, Prusinkiewicz *et al.* (2009) demonstrated that the mechanistic basis for an indirect action of auxin on bud inhibition could be the positive feedback between auxin flux and polarization of active auxin transport. Furthermore, these authors, based on an L-system modelling exercise, found that the assumption of saturation of auxin transport capacity in the main stem was not necessary for auxin transport inhibition of branching. In acknowledgement of this finding, the theory was renamed as the canalization hypothesis (Leyser, 2009). This hypothesis is based on auxin transport and canalization of auxin transport pathways from axillary buds into

the main stem, which acts as a sink for auxin. If the sink strength in the main stem is strong, then canalization of the auxin efflux pathway from buds will occur and stimulate bud outgrowth, whereas weak sink strength prevents such canalization and growth is prevented. Additionally, Liang *et al.* (2010) found in chrysanthemum (*Dendranthema grandiflorum*) that strigolactones only effectively inhibited bud outgrowth when in the presence of a competing auxin source, which supports the hypothesis that strigolactones inhibit bud outgrowth by modulating auxin transport canalization. Crawford *et al.* (2010) have since confirmed this finding and shown that strigolactones act by damping auxin transport, thereby enhancing competition between developing branches. Auxin was also found to mediate the feedback by strigolactone on strigolactone biosynthesis (Liang *et al.*, 2010).

The regulation of branching in white clover (*Trifolium repens* L.), a perennial nodally rooting prostrate-stemmed herb, differs from that of the annual erect-stemmed species of *Arabidopsis thaliana*, *Pisum sativum*, and *Petunia hybrida* in which apical dominance plays a major role. In contrast, in *T. repens* the regulatory processes are dominated by a net root-derived stimulatory signal (NRS) that is transported acropetally in the vascular tissues (Thomas and Hay, 2008, 2009). Sectorial responses in the shoot immediately distal to nodal roots, and the predominantly acropetal transport of NRS from them (Thomas and Hay, 2007), suggest that this transport initially occurs in the transpiration stream.

When a stem cutting of *T. repens* is rooted only at its base, it is unable to sustain continued branching along the stem, and the rate of development of successively produced axillary buds declines from node to node (Thomas *et al.*, 2002). The primary factor driving this decline in bud outgrowth is hypothesized to be the decline in NRS availability throughout the shoot system distal to a nodal root concomitant with the continued development of the shoot (Thomas and Hay, 2008). It is suggested that this decline in NRS results because the increase in NRS production by roots does not match the increasing demand for it by the ever-enlarging shoot system and that this leads to the signal becoming increasingly scarce within the shoot. This decline in NRS availability is significant because the activation level (growth rate) of any particular axillary bud is related to the NRS available to it immediately following its emergence from its parent apical bud. This activation level is then retained for at least 6 weeks (Thomas and Hay, 2007).

Excision of any number of branches distal to a nodal root promotes the outgrowth of a corresponding number of buds further along the stem so that ultimately the same complement of branches is formed (Thomas *et al.*, 2003a). Thus it is the number of elongating intact branches intercalated between a nodal root and a distal axillary bud that determines the likelihood of outgrowth of that bud (Thomas *et al.*, 2003a; Thomas and Hay, 2009). The question is thus raised as to possible mechanisms by which previously formed basal branches inhibit the outgrowth of axillary buds distal to them.

The possibility that the excision of buds/branches precipitates a 'non-auxin fast decapitation signal' that primes axillary buds for growth as described for pea (Morris *et al.*, 2005; Ferguson and Beveridge, 2009) seems unlikely. In *T. repens*, axillary buds are already 'primed for growth' when they emerge from their parent apical bud. At this stage they are actively producing leaf primordia at the same rate as the apical bud (Thomas, 1962) and this growth rate is then up- or down-regulated according to NRS availability (Thomas and Hay, 2007). Furthermore, during the frequent use of decapitation as a manipulative treatment of primary and branch stems (Thomas *et al.*, 2003b; Thomas and Hay, 2007, 2008, 2009), no response in basally positioned axillary buds well distanced from the decapitation site similar to that found in pea (Morris *et al.*, 2005; Ferguson and Beveridge, 2009), or consistent with these treatments initiating out-growth responses via their disturbances of the hydraulic conductivity of tissues (McIntyre, 1987), has been observed.

The major possible mechanisms of control by basal branches can be simplified down to two distinctly different possibilities in which these branches either feed a basipetally transported inhibitory influence into the shoot system via the symplast or function as sinks for a stimulatory root-derived bud activation signal (NRS) transported via the apoplast. These possibilities in turn give rise to the following three hypotheses, or combinations thereof, as mechanisms of control: (I) the apical buds on branches produce a phloem-mobile inhibitory signal, such as the branch-derived feedback signal proposed by Dun *et al.* (2009b), that is transported down the branch and then moderates the synthesis of branching signals for acropetal movement in the primary stem; (II) the polar flow of auxin from branches limits further branch development by moderating branching signals such as cytokinin and strigolactone synthesized within the root or stem system that in turn regulate bud outgrowth as suggested in the second messenger (Snow, 1937) or the auxin transport canalization (Leyser, 2009; Crawford *et al.*, 2010) theories; or (III) branches function as sinks for a root-derived stimulatory signal (NRS) thereby decreasing its availability to more distally located axillary buds. Experiments were designed to test the first two hypotheses and to determine whether responses were consistent with the third remaining as a possibility for further testing.

Materials and methods

Plant material

Trifolium repens L. (white clover) plants used in both experiments were derived from a greenhouse-grown stock clone of a single genotype selected from a Spanish ecotype collection (AgResearch Accession number C1067) as previously described (see Thomas *et al.*, 2003a; Thomas and Hay, 2007, 2008).

Hormone materials

To produce the hydrous lanolin paste, anhydrous lanolin was melted before water was added in the ratio 3:2 (lanolin:water) by weight, and the mixture was vigorously stirred.

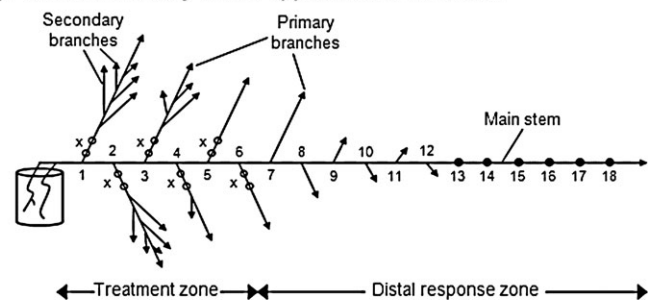
For the NAA lanolin paste, 1-naphthaleneacetic acid (NAA) dissolved in a drop of ethanol was added to the molten hydrated lanolin paste (10 mg of NAA g⁻¹ hydrated lanolin) and stirred so as to mix thoroughly.

For the NPA lanolin paste, NPA dissolved in ethanol was added to the molten hydrated lanolin paste (10 mg of NPA g⁻¹ hydrated lanolin) and stirred so as to mix thoroughly.

Culture of experimental plants

Plants were grown from stem tip cuttings planted on 1 July 2009 (Experiment 1) and 14 January 2010 (Experiment 2) in a commercially obtained potting mix (Thomas *et al.*, 2002) in 1.8 l plastic pots. After ~3 weeks, the two or three basalmost branches formed by this time were trimmed off each plant to leave a single stem axis growing away from its basal root system. All lateral branches that grew out subsequently from this main stem were retained. The oldest phytomer on the main stem that retained a branch at its node was termed phytomer 1 (P1) and later formed ones were termed P2, P3, etc. (Fig. 1A). Outgrowth of nodal roots was prevented by growing shoot systems out over a dry plastic mesh. Throughout both experiments, plants were grown in a heated

(A) Plants immediately before application of treatment



(B) Treatments applied to basal branches in the treatment zone

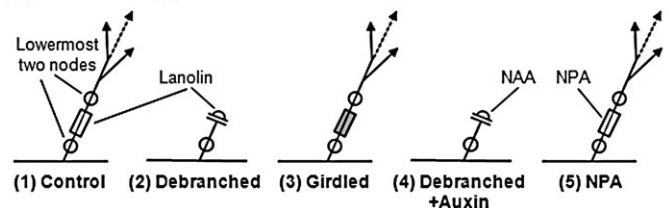


Fig. 1. The design and experimental procedures followed in Experiment 1. (A) State of the plants at the time of treatment application showing the six treated basalmost branches (treatment zone, phytomers labelled 1–6) with sites of treatment applications indicated by an X, and the distal portion of the plant (response zone, phytomer 7 onwards) in which the response to treatment was assessed. (B) For each treatment, a diagram of one of the six identically treated basalmost branches for control, debranched, girdled, debranched+auxin, and NPA auxin transport inhibitor treatments. Numbers indicate phytomer positions on the main stem, solid arrowheads indicate shoot apical buds, black dots represent emerged nodes bearing axillary buds, open circles depict the nodes from which axillary buds and leaves were removed, and short double lines indicate points of stem excision at which lanolin was applied with or without the addition of auxin. In (B) the shaded oblong shows the position of the wax girdle and the open oblongs the positions at which lanolin with or without NPA was applied around stems.

greenhouse in natural photoperiods at average maximum/minimum temperatures of 25/12 °C.

Methods

Experiment 1: This experiment was designed to test the validity of each of the three hypotheses proposed in the Introduction as mechanisms by which established branches might regulate the development of axillary buds distal to them.

When the branches at P1–P6 had formed ≥ 6 expanded leaves, the leaves and axillary buds at the oldest, basalmost, two nodes of each were excised to provide an unobstructed surface on which to apply the experimental treatments. Plants then were grown on until their main stems had 16–18 expanded leaves and 9 or 10 elongating primary branches (Fig. 1A), at which time, 15 October 2009, treatments were imposed. Plants were assigned to replicates such that uniformity within replicates was maximized and then randomly assigned to treatments within replicates.

Treatments were applied to the middle of the second internode (the internode distal to the first node) on each of the six primary branches formed at P1–P6 in the treatment zone of the primary stem (see Fig. 1), leaving the remainder of the plant untreated. The effects of these treatments on the development of axillary buds on the branches and on the main stem at and distal to P7 (the distal response zone) were then assessed over a 2 week period and the responses used to identify the most likely regulatory mechanism (see Table 1).

Treatments given were as follows (Fig. 1B).

- (1) Control: plants were untreated except for the application of hydrous lanolin paste to the second internode of each of the first six basal branches [Fig. 1B, (1)] as a control for treatment 5, below.
- (2) Debranched: the six oldest basal branches were each excised distal to their lowest node by cutting through the middle of their second internode [Fig. 1B, (2)]. Hydrous lanolin paste was applied to the ends of the remaining branch stumps as a control for treatment 4, below. This treatment prevented the possible export of phloem-mobile signals (Hypothesis I) and polar auxin flow (Hypothesis II) from them and it prevented the functioning of basal branches as sinks for transported NRS (Hypothesis III).
- (3) Girdled: molten candle wax heated to 110 °C was applied via a custom-built Perspex chamber to a 10 mm segment of the second internode on each of the six oldest basal branches (Fig. 2). Transmission electron microscopy (see Supplementary Fig. S1 available at *JXB* online) verified that this treatment kills all living cells in the girdled stem segments, thereby preventing all symplastic transport out of the branches back into the primary stem (Hypotheses I and II) while leaving branches present to receive xylem-transported substances inclusive of NRS (Hypothesis III) (Snow, 1929; Davies and Wareing, 1965; van Kleunen and Stuefer, 1999).
- (4) Debranched+auxin: the six basal branches were excised as in treatment 2 but NAA was applied in lanolin paste to the cut ends of the branch stumps [Fig. 1B, (4)]. This treatment

prevented the possible export of phloem-mobile signals (Hypothesis I) and the possibility of basal branches receiving NRS (Hypothesis III), but allowed the possibility of polar flow of auxin from branch stumps back into the main stem (Hypothesis II) by substituting a source of auxin at their cut ends.

- (5) Auxin transport inhibitor: NPA was applied in lanolin paste around a 10 mm segment of the second internode of each of the six basal branches [Fig. 1B, (5)]. This treatment was expected to prevent the polar flow of auxin out of the branches (Hypothesis II) while allowing export of all other phloem-mobile compounds from them into the main stem (Hypothesis I) and the possibility for branches to function as sinks for NRS (Hypothesis III).

In all treatments, the lanolin-based applications were reapplied twice weekly. A randomized block design was used for the experiment with the five treatments replicated eight times.

The developmental state of each plant at the time experimental treatments were imposed was assessed by measuring the lengths of all branches and main stems and counting the number of emerged leaves on each using the Carlson scale of leaf development (Carlson, 1966). Lengths and leaf numbers were then reassessed 7 d after treatment in the portion of the plant distal to the six treated branches (i.e. in the distal response zone from P7 onwards, Fig. 1), and again after 14 d in the whole plant. Shoots were then divided into three portions: the six branches in the basal treatment zone (Fig. 1A); all branches in the distal response zone from P7 onwards; and the whole of the main stem including its leaves. Dry weights of these portions were then determined after drying to constant weight for 4 d in a draught oven at 60 °C.

Experiment 2: This experiment was designed (i) to test whether the auxin (NAA) and auxin transport inhibitor (NPA) treatments as applied in the previous experiment induced morphological responses consistent with them effectively altering auxin transport within *T. repens* and (ii) to assess their involvement in the responses to decapitation of stems.

The hormone pastes used in this experiment were prepared as for Experiment 1 and reapplied to plants twice weekly. Cuttings were grown on until their main stems had 12 fully expanded leaves, at which time (6 March 2010) the following treatments were applied to the internode distal to the 12th leaf.

- (1) Control: in which the main stem was left intact and lanolin paste was applied around a 10 mm section of the internode distal to leaf 12.
- (2) Decapitation: stem apical tissues were excised distal to the leaf and axillary bud at P12 and lanolin paste was applied to the cut surface of the stem.
- (3) Decapitation+NAA: decapitated as in treatment 2 and NAA supplied in lanolin paste applied to the cut stem surface.
- (4) NPA: the main stem remained intact and NPA in lanolin paste was applied to a 10 mm section of the internode distal to leaf 12.

Table 1. For Experiment 1, the suite of positive branching responses in the distal response zone of the shoot that is predicted to occur if Hypothesis I, II, or III is correct

Hypothesis	Treatment				
	Control	Debranched	Girdled	Debranched+auxin	NPA
I. Phloem-mobile inhibitor	–	✓	✓	✓	–
II. Auxin transport	–	✓	✓	–	✓
III. Branches are sinks for NRS	–	✓	–	✓	–

Each plant was assessed at the time of treatment and again 7 d and 14 d after treatment by measuring the length of all buds/branches and counting the number of emerged leaves using the Carlson scale of leaf development (Carlson, 1966). Each treatment was replicated eight times and the trial finished on 1 April 2010.

Analysis of data

For Experiment 1, axillary buds and branches were identified by the position of their phytomer of origin on the main stem or on any other stem of higher branching order. Branches originating from the main stem were termed primary branches, those originating from them secondary, and those originating from secondary branches, tertiary.

The dry weight data in Table 2 were analysed by one-way analysis of variance (ANOVA) within the Excel 2007 package. All other data relating to growth of branching stems were analysed in R software (R Development Core Team, 2009) by ANOVA using a generalized linear mixed model with treatment as a 'fixed effect' and replicate as a 'random effect' within the package 'nlme' (Pinheiro *et al.*, 2009), except for the data set for stem length of secondary branches in the response zone which contained many

zero values and lacked a normal distribution. This data set was analysed using the generalized linear mixed model with Poisson distribution in the package 'lme4' (Bates and Maechler, 2009). In all cases, the treatment means generated by the appropriate model along with the associated $LSD_{5\%}$ are presented in the tables and in Fig. 3.

For Experiment 2, the length of the axillary bud stems forming at phytomer positions 9–12 for all four treatments were separately analysed at each phytomer position for treatment differences by one-way ANOVA in GenStat (Payne *et al.*, 2007). At P13, as the lengths of the axillary bud stems in the control and NPA treatments were both approaching zero (Fig. 4), no tests were undertaken.

Results

Experiment 1: shoot dry weight

The control, girdled, and NPA treatments had similar dry weights for all three plant portions (Table 2). The distal branch dry weights in the debranched and debranched+auxin treatments were significantly ($P < 0.05$) greater than in the other three treatments. Dry weight of the main stem in the debranched treatment was greater ($P < 0.05$) than that of the girdled treatment.

Experiment 1: growth and branching of the six basalmost treated branches (treatment zone)

Growth of the six girdled stems was slightly reduced relative to that in the control and NPA treatments. The increments of growth over the 14 d experimental period on the six treated basal primary branch stems of the control, girdled, and NPA treatments as assessed by the summed increase in length and total number of new leaves to emerge on them were 527, 313, and 436 mm ($LSD_{5\%}$ 153.0) and 20.4, 16.7, and 19.7 ($LSD_{5\%}$ 2.72), respectively.

The increase in number of secondary and tertiary branches developing on the six treated basal primary branch stems over the 14 d experimental period did not differ significantly among the control, girdled, and NPA treatments (Table 3). An apparent 30% decrease in elongation of girdled branches relative to control and NPA treatments was not statistically significant.

Experiment 1: growth and branching in the distal response zone tissues (P7 onwards)

The main stem distal to the treated branches: Over the 14 d response period the main stem produced more leaves in the

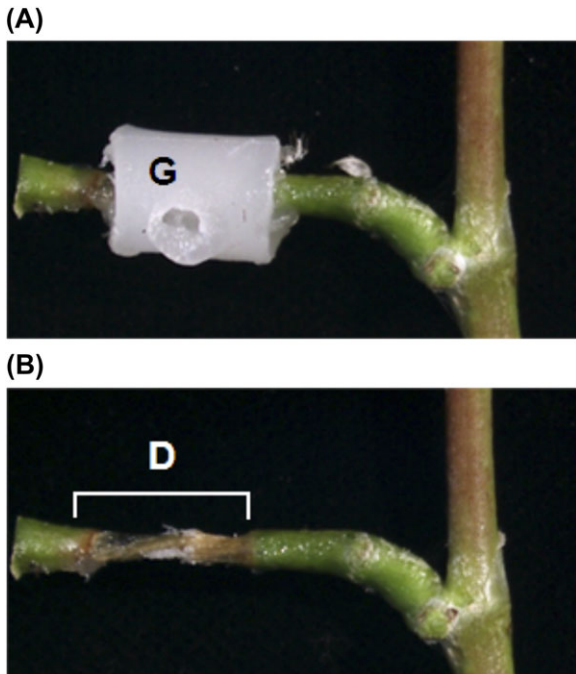


Fig. 2. Photographs of (A) a wax girdle (G) in place on a stem and (B) the same stem after removal of the wax girdle at the end of Experiment 1 showing the zone of dead tissue (D) and a slight increase in stem diameter distal to the girdle.

Table 2. Dry weights (g) of the three shoot portions (stem plus leaves) of plants of each treatment at the end of Experiment 1; $n=8$

Shoot portion	Treatment					$LSD_{5\%}$	F ratio
	Control	Debranched	Girdled	Debranched+auxin	NPA		
Six treated branches	17.32	–	19.41	–	17.94	4.230	0.59 NS
All distal branches	2.44	3.61	1.98	3.42	2.49	0.592	1.60***
Main stem	1.56	1.71	1.42	1.62	1.53	0.203	2.47 NS

NS, not significant; *** $P < 0.001$.

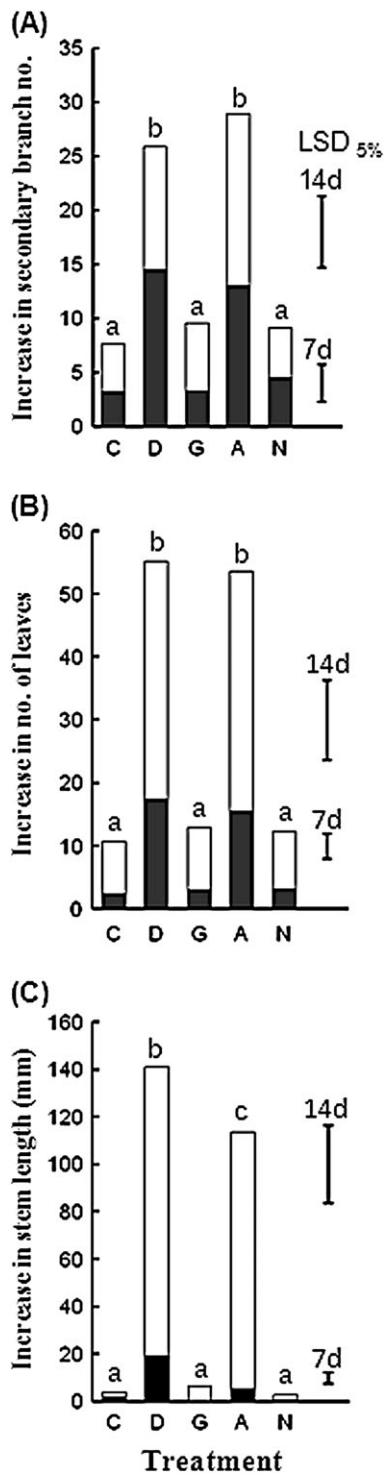


Fig. 3. Effects of treatments on secondary branch development in the distal response zone of plants in Experiment 1. Bar graphs show the increases in (A) the number of secondary branches, (B) the total number of leaves on them, and (C) their total stem lengths (mm) after 7 d (filled) and 14 d (open) of treatment. Treatments are: control (C), debranched (D), girdled (G), debranched+auxin (A), and NPA (N). Thin bars on the right represent the $LSD_{5\%}$ for 7 d or 14 d. Different letters above wide bars indicate treatment differences ($P < 0.05$) for the 14 d values.

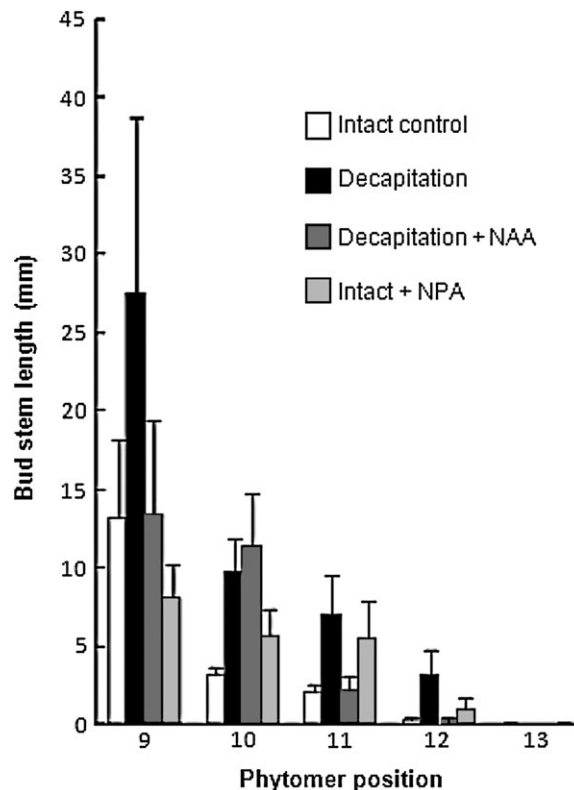


Fig. 4. The length of the axillary bud stems (mm) after 14 d of treatment in Experiment 2. The treatments are: control, decapitation distal to node 12, decapitation distal to node 12 with NAA application, and NPA applied to the internode distal to node 12 on intact plants. Bars represent the SE of the means, $n=8$.

Table 3. The increase on the six basalmost treated branches (at P1–P6) in total number and length of secondary branches (2°) and in the number of leaves on them over the 14 d experimental period, and the number and length of tertiary branches (3°) at the end of Experiment 1 in the control, girdled, and NPA treatments

	Treatment			$LSD_{5\%}$
	Control	Girdled	NPA	
2° branches				
Number	15.6	16.4	17.1	4.64
Length (mm)	1399	1035	1283	373.7
No. of leaves	76.4	72.5	78.9	17.91
3° branches				
Number	41.9	42.0	44.3	19.04
Length (mm)	254	197	289	204.3

Means are presented, $n=8$, along with the $LSD_{5\%}$ obtained from the appropriate ANOVA.

debranched and debranched+auxin treatments than in the control, girdled, and NPA treatments. Girdled plants produced fewer leaves than controls (Table 4). The increase in length of the main stem over the 14 d period in the debranched treatments, both with and without auxin, was approximately double that of the control, girdled, and NPA treatments. These trends were already evident after 7 d.

Table 4. For Experiment 1, the increase in leaf number and stem length of the main stem and increase in number and length of primary branches and number of leaves on them in the distal response zone of plants from phytomer 7 onwards in response to treatments after 7 d and 14 d

		Treatment					LSD _{5%}
		Control	Debranched	Girdled	Debranched+auxin	NPA	
Main stem							
Number	7 d	1.12	1.54	0.77	1.52	0.91	0.453
	14 d	2.72	3.46	1.86	3.59	2.35	0.672
Length (mm)	7 d	20	34	13	31	16	18.1
	14 d	42	66	25	83	28	30.0
Primary branches							
Number	7 d	1.12	2.38	0.75	2.75	0.50	1.090
	14 d	2.12	4.75	1.50	5.38	1.88	1.389
Length (mm)	7 d	121	173	74	165	108	48.7
	14 d	239	429	132	422	210	99.5
No. of leaves	7 d	7.0	13.0	5.5	12.4	7.3	2.13
	14 d	15.7	27.7	12.1	29.0	16.9	3.10

Means are presented, $n=8$, along with the LSD_{5%} obtained from the appropriate ANOVA.

Outgrowth of axillary buds along the main stem to form primary branches was also stimulated by debranching. Both the number of new buds showing leaf emergence after 14 d and their length at that time were greater ($P < 0.05$) in debranched plants than in control, girdled, and NPA treatments. Bud outgrowth in response to NPA treatment was similar to that in control plants, but girdled plants gave a lower value than the control treatment (Table 4). This trend was also evident after 7 d of treatment.

Experiment 1: development of the distal primary branches (P7 onwards)

The increases in the length of primary branch stems from P7 onwards, and in the total number of leaves on them, in the control, girdled, and NPA treatments were approximately half those of the debranched and debranched+auxin treatments after both 7 d and 14 d of treatment (Table 4). Values for the girdled treatment were significantly lower than those of the control treatment. The increase in number of secondary branches formed on these primary branches and in the number of leaves on them, and in their length over both 7 d and 14 d of treatment, were all similar in the control, girdled, and NPA treatments. These were approximately only one-third and 5%, respectively, of the values for the number and stem length recorded in the debranched and debranched+auxin treatments after 14 d, however (Fig. 3).

Experiment 2

Decapitation relative to the control treatment increased bud stem length at all four of the youngest remaining phytomers (P9–P12), significantly so at P10–P12 (Fig. 4). Application of NAA to the decapitated stump decreased bud lengths at P11 and P12 relative to the decapitated treatment, so that they matched those of the untreated controls. No significant

influence of NAA in reversing the effect of decapitation was apparent at P9 and P10, however. For phytomer positions 10, 11, and 12, but not at P9, there was a consistent trend for the NPA treatment to increase bud stem lengths relative to the control treatment.

Discussion

The response pattern predicted by Hypotheses I and II for a regulatory influence on axillary bud outgrowth via translocation of either a phloem-mobile inhibitory signal or of auxin out of branches included a positive response to the girdled treatment (Table 1) as this would have prevented their efflux from branches by killing all living cells in the treated stem segment. This, however, was not observed; outgrowth of axillary buds to form secondary branches in the distal response zone in the girdled treatment did not differ significantly from that in the control treatment (Table 4; Fig. 3). The debranched+auxin and NPA treatments were included to distinguish between the possible action of auxin transport mechanisms and of a non-auxin phloem-mobile inhibitory signal should a positive response to the girdled treatment have been observed. The lack of any positive branching response to the girdled and NPA treatments, together with the failure of exogenously supplied auxin to down-regulate the large branching response to debranching in the debranched+auxin treatment, indicates that neither polar auxin flow nor phloem-mobile signal movement out of branches (Dun *et al.*, 2009b) plays any significant part in the correlative inhibition of distal branching by established proximal branches in *T. repens*. It is most unlikely that the lack of responses to applications of NAA and NPA in Experiment 1 was caused by the failure of the lanolin pastes to deliver biologically meaningful quantities of hormone: the results of Experiment 2 (Fig. 4)

suggest that both these treatments were effective in manipulating auxin flow within *T. repens* stems. That polar auxin flow did occur in Experiment 1 is indicated by a slight thickening of branch stems that was manifest immediately distal to their girdles (Fig. 2) in a similar way to that described by Ferguson and Beveridge (2009) as a result of auxin accumulation in *Pisum*. An additional consequence of girdling was that the supply of carbon from the large basal branch systems back into the main stem and roots would also have been blocked. This probably accounted for the non-significant trends for increased dry weight of basal branches, the decreased distal branch dry weight, and the tendency for there to be reduced leaf appearance rates and lengths of the main stems and distal primary branches in the girdled as opposed to the untreated control plants (Tables 2, 4). Thus even though these intra-plant adjustments to accommodate an altered carbon balance were occurring in girdled plants, they did not significantly influence the outgrowth of higher order branches either on the treated basal primary branches (Table 3) or in the distal response zone over the 14 d response period (Table 4; Fig. 3).

Two key findings were consistent in supporting the alternative hypothesis (Hypothesis III) that correlative inhibition of branching in distal plant parts results from existing branches functioning as sinks for a root-derived branching promoter (NRS). The first of these, that growth and branching on the girdled P1–P6 branches continued at rates approaching those in the control treatment (Table 3), indicates that delivery of NRS to them was sufficient for branch development and provides direct evidence that the transport of NRS into them occurs predominantly via the xylem (Thomas and Hay, 2008). This also demonstrates the continued functioning of xylem within the girdled zone (Snow, 1929). Secondly, the strong branching response of the main stem and primary branches in the distal response zone to basal debranching (Table 4; Fig. 3), with or without applied auxin, is consistent with the distribution of NRS to the distal portion of the plant that would otherwise have been allocated to the P1–P6 basal branches (Thomas *et al.*, 2003a; Thomas and Hay, 2007, 2008).

The nature of the mechanism by which debranching stimulates bud outgrowth in distal regions of the shoot is uncertain. Bearing in mind the confirmation in Experiment 1 that long-distance transport of NRS in *T. repens* is via the xylem, one obvious possibility is that excision of basal branches led to increased availability of NRS in the distal region of the main stem as a result of this region now being the only remaining transpiring portion of the shoot. As frequently observed in other species (Else *et al.*, 1995; Siebrecht *et al.*, 2003), it is the rate of loading of solutes into the xylem in the root system, rather than the transpiration rate, that determines their rate of delivery to the shoot system. As a result, solute concentrations in xylem sap will vary with changes in transpiration rates. That being so, and assuming an unchanged rate of loading into the xylem, the decreased volume of xylem sap in the whole shoot consequent upon basal branch excision would be expected to lead to an increased concentration of NRS in

the sap. Thus, all of the NRS loaded into the xylem would now be delivered to the distal region, very probably at an increased concentration that could lead to the observed boost to outgrowth of distal axillary buds.

The long-distance movement of NRS signal within the shoot might not always necessarily be solely via the xylem, however. This is indicated, for instance, by the results of an experiment designed to demonstrate the relationship between transpiration rate and axillary bud outgrowth in *T. repens* (see Supplementary Table S1 at *JXB* online). In this experiment, in which basal branches were enclosed in clear plastic bags fastened so as to reduce transpiration severely, with the remainder of the plant left untreated, it was predicted that reduced transpiration of the basal bagged branches would restrict flow of xylem sap into them and reduce bud outgrowth on them by reducing delivery of NRS. As a result, bud outgrowth in the freely transpiring distal region of the shoot would be stimulated in response to its receipt of almost all the root-synthesized NRS loaded into the xylem. This did not happen; axillary bud outgrowth on the basal branches continued despite reduced transpiration and there was no increase in bud outgrowth in the distal shoot portion. This suggests relatively ready delivery of NRS to the buds of basal branches by mechanisms other than by movement in the transpiration stream. Significantly, in addition, when basal branches are ‘disbudded’ (Thomas *et al.*, 2003a) by removing just their actively growing axillary and apical buds, the large bud outgrowth response in the shoot distal to the ‘disbudded’ branches is very similar to that observed when whole branches are excised (Thomas *et al.*, 2003a). This is so despite the impact on branch transpiration being minimal and the delivery of NRS into branches via the xylem therefore being little affected. Results from Experiment 1 in this study indicate that this response was not driven by decapitation effects on the movement of auxin or any phloem-mobile signals from these branches. Thus another possibility is that the NRS delivered to the ‘disbudded’ basal branches, but not utilized in bud outgrowth, is returned to the main stem possibly in the phloem. Hence it would appear that NRS is synthesized in roots, where it is loaded into the xylem, and that initial transport from roots to the shoot is via the xylem in much the same way as mineral nutrients are initially transported via the xylem. However, once in the shoot, it appears that other transport mechanisms are available for its distribution. These possibilities remain to be tested.

The nature of the NRS signal is currently unknown. The known promoters of axillary bud outgrowth are cytokinin (Ferguson and Beveridge, 2009) and a recently documented root-synthesized signal which was found when either the *CCD7/DAD3* or *CCD8/DAD1* genes were mutated in *Petunia* (Drummond *et al.*, 2009; Janssen *et al.*, 2010). Results from the present study are not able to distinguish between these possibilities. It is unlikely that nutrients are the NRS signal involved as additional nutrient supplied in a foliar spray to non-rooted shoot portions stimulates both the rate of growth and size of shoot organs but fails to restore axillary bud outgrowth in the non-branching shoot

zone of *T. repens* plants (Hay *et al.*, 2003). A predicted reduction in supply of auxin to roots, brought about by girdling or auxin transport inhibition in the NPA treatment, did not increase axillary bud outgrowth even though such a reduction in auxin would have been expected to up-regulate the synthesis of root-derived cytokinin (reviewed by Shimizu-Sato *et al.*, 2009). However, an increase in root synthesis of xylem-transported cytokinin does not necessarily increase shoot branching (Faiss *et al.*, 1997), and the importance of local shoot-synthesized cytokinin for axillary bud activation has recently been emphasized (Nordström *et al.*, 2004; Tanaka *et al.*, 2006; Dun *et al.*, 2009a; Ferguson and Beveridge, 2009). It has been demonstrated recently in pea, however, that xylem sap cytokinin has a role in sustaining the outgrowth of buds after they have initiated growth but no role in the initiation of bud outgrowth (Beveridge *et al.*, 2009; Dun *et al.*, 2009b). Thus given that in *T. repens* the axillary buds emerge from their parent apical buds already actively growing (Thomas, 1962; Thomas *et al.*, 2003b), the possibility for involvement of root-synthesized cytokinin in the NRS signal cannot be ruled out.

The results of the present study reaffirm the significance of a root-derived branching promoter signal in the regulation of branching in *T. repens* by centring on the mechanism by which the inhibitory influence of basal branches is asserted. They therefore lend strong support to the suggestion that a more comprehensive understanding of the regulation of branching in general might ensue following the characterization of a root-derived positive signal for branching (Drummond *et al.*, 2009; Janssen *et al.*, 2010).

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Light microscopy of cross-sections of control and girdled stems.

Table S1. The effect of manipulation of the rate of transpiration of basal branches on shoot branching.

Acknowledgements

We thank Paul Lovejoy for designing and constructing the purpose-built Perspex chamber used to apply molten candle wax to the stems of plants, Rachael Sheridan for technical assistance, John Koolaard and Dongwen Luo for statistical advice, and two anonymous referees for insightful and constructive comments on earlier drafts. This work was funded by the MeriNet programme, New Zealand Foundation for Research, Science and Technology, contract C10X0816.

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