Antagonistic Action of Strigolactone and Cytokinin in Bud Outgrowth Control[1][W]

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Cytokinin (CK) has long been implicated as a promoter of bud outgrowth in plants, but exactly how this is achieved in coordination with other plant hormones is unclear. The recent discovery of strigolactones (SLs) as the long-sought branch-inhibiting hormone allowed us to test how CK and SL coordinately regulate bud outgrowth in pea (*Pisum sativum*). We found that SL-deficient plants are more sensitive to stimulation of bud growth by low concentrations of locally applied CK than wild-type plants. Furthermore, in contrast with SL mutant plants, buds of wild-type plants are almost completely resistant to stimulation by CK supplied to the vasculature. Regardless of whether the exogenous hormones were supplied locally or to the xylem stream, SL and CK acted antagonistically on bud outgrowth. These data suggest that SLs do not affect the delivery of CK to axillary buds and vice versa. Rather, these data combined with dose-response experiments suggest that SLs and CK can act directly in buds to control their outgrowth. These hormones may converge at a common point in the bud outgrowth regulatory pathway. The expression of pea *BRANCHED1*, a TCP transcription factor expressed strongly in buds and thought to act downstream of SLs in shoot branching, is regulated by CK and SL without a requirement for protein synthesis and in a manner that correlates with observed bud growth responses.

Shoot branching is a major determinant of plant shoot architecture. Many factors contribute to the ability of an axillary bud to grow out to form a branch, including developmental, positional, genetic, hormonal, and environmental factors. Auxin, cytokinin (CK), and strigolactones (SLs) are implicated in the hormonal regulation of bud outgrowth; auxin and SLs as inhibitors of bud outgrowth and CK as a promoter of bud outgrowth (Dun et al., 2009a; Leyser, 2009; Beveridge and Kyozuka, 2010). Many studies over a number of decades have investigated the antagonistic action of auxin and CK in bud outgrowth control (Shimizu-Sato et al., 2009) and, more recently, the relationships between auxin and SL (Brewer et al., 2009; Crawford et al., 2010; Liang et al., 2010), but how SL and CK integrate to antagonistically control bud outgrowth remains unclear.

Prior to their identification as hormones involved in shoot branching, certain properties of SLs were characterized based on studies of the long-distance branch-inhibiting signal in a series of increased branching mutants. These mutants include *ramosus* (*rms*) in pea (*Pisum sativum*), *more axillary growth* (*max*) in Arabidopsis (*Arabidopsis thaliana*), *decreased apical dominance* (*dad*) in *Petunia hybrida*, and *dwarf* (*d*) and *high tillering dwarf* (*htd*) in rice (*Oryza sativa*; for review, see Dun et al., 2009a; Beveridge and Kyozuka, 2010; Domagalska and Leyser, 2011). Grafting studies demonstrated that the branch-inhibiting signal can be synthesized in root or shoot tissue, moves upward to inhibit bud outgrowth, and that a subset of the branching mutants are unable to synthesize the signal (now named SL synthesis mutants; *rms1/max4/dad1*, *rms5/max3/dad3*, *max1*), while others are unable to respond to it (SL-insensitive mutants; *rms4/max2*, *rms3*, *dad2*; Beveridge et al., 1996, 1997b; Napoli, 1996; Morris et al., 2001; Turnbull et al., 2002; Sorefan et al., 2003; Booker et al., 2005; Simons et al., 2007). These studies also indicated that the branch-inhibiting signal or its precursor(s) may be translocated over long distances and may act locally in, or near, axillary buds. These characteristics hold true for SLs; treatment of SL in hydroponics, growth medium, or supply of SL to the stem or bud can inhibit bud outgrowth in the SL synthesis mutants but not in the SL-insensitive mutants (Gomez-Roldan et al., 2008; Umehara et al., 2008; Dun et al., 2009b).

The SL biosynthesis pathway is gradually being pieced together. SLs are derived from the carotenoid pathway; a carotenoid precursor (likely β-carotene) is sequentially cleaved by CAROTENOID CLEAVAGE DIOXYGENASE7 (CCD7) and CCD8, encoded by *RMS5/MAX3/DAD3/HTD1/D17* and *RMS1/MAX4/*

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DAD1/D10, respectively (Xie et al., 2010). D27 is a novel iron-containing protein that likely acts in the plastid after CCD7 and CCD8 (Lin et al., 2009). Grafting studies indicate that *MAX1*, encoding a cytochrome P450, then acts on a mobile intermediate (Booker et al., 2005). *D14* (encoding an α/β-fold hydrolase) and *RMS4/MAX2/D3* (encoding an F-box protein) may function downstream of SL synthesis and are required for response to SLs (Stirnberg et al., 2002; Ishikawa et al., 2005; Johnson et al., 2006; Arite et al., 2009; Gao et al., 2009; Liu et al., 2009).

Auxin originating from the shoot tip has long been known to repress the outgrowth of axillary buds located at nodes below (Thimann and Skoog, 1933, 1934). However, since auxin moves strictly downward in the polar auxin transport stream and does not enter the axillary buds that it inhibits, second messengers for auxin were proposed (for review, see Dun et al., 2009a; Leyser, 2009). Prior to studies with the SL increased-branching mutants, CK was a candidate antagonistic second messenger for auxin (for review, see Cline, 1991). Evidence for this includes that auxin negatively regulates the expression of CK biosynthesis genes (*Adenosine Phosphate-Isopentyltransferase* [*IPT*] genes) and positively regulates the expression of CK metabolism genes (Nordström et al., 2004; Tanaka et al., 2006; Werner et al., 2006; Shimizu-Sato et al., 2009). Additionally, removal of auxin via decapitation of the shoot tip leads to increased CK levels, which are restored by auxin treatment (Bangerth, 1994; Shimizu-Sato et al., 2009).

Prior to the discovery of SL as a branching hormone, decapitation studies with the *rms* increased-branching mutants demonstrated that the branch-inhibiting signal is required for indole-3-acetic acid (IAA; an auxin) inhibition of decapitation-induced branching (Beveridge et al., 2000) and that auxin positively regulates the expression of the branching inhibitor biosynthesis genes (*RMS1* and *RMS5*; Foo et al., 2005; Johnson et al., 2006). These findings are corroborated by gene expression studies in rice and Arabidopsis (Zou et al., 2006; Arite et al., 2007; Hayward et al., 2009) and indicate that SL may act as a second messenger for auxin. Indeed, SL treatment to axillary buds of decapitated pea plants prevents decapitation-induced outgrowth of the treated bud without the need for an apical auxin source (Brewer et al., 2009). However, IAA treatment to the apical end of Arabidopsis or chrysanthemum (*Dendranthema grandiflorum*) isolated nodal segments appears necessary for bud growth repression by basal supply of SL (Crawford et al., 2010; Liang et al., 2010). The reason for these differences in response to SL in different experimental situations is unclear. The contribution of differing CK levels to these results remains to be considered; the effectiveness of SL treatments may depend on relative CK content.

CK was implicated in shoot-branching control when exogenous applications of CKs to axillary buds were shown to stimulate their outgrowth (Sachs and Thimann, 1967). While it is likely that the regulation of local CK biosynthesis may be an important regulator of bud outgrowth (Böhner and Gatz, 2001; Tanaka et al., 2006; Ferguson and Beveridge, 2009; Shimizu-Sato et al., 2009), the exact roles of local versus long-distance CK in the modulation of bud outgrowth remain unclear. Some studies suggest that only local CK promotes bud outgrowth (Faiss et al., 1997), whereas for *rms* SL mutants, we recently proposed that xylem sap CK promotes sustained branch growth rather than functioning in bud release (Dun et al., 2009b). This hypothesis would account for earlier work that showed no branching promotion in wild-type shoots with an intact shoot tip in response to enhanced xylem sap CK (Faiss et al., 1997). In pea, buds that are released from inhibition by SLs grow longer in the presence of elevated xylem sap CK levels (Dun et al., 2009b). The growth of activated wild-type Arabidopsis buds on isolated nodal segments in split-plate assays is accelerated by basal treatment with 6-benzylaminopurine (BA; synthetic CK) to the medium (Chatfield et al., 2000). Consequently, our hypothesis is that xylem sap CK may stimulate the continued outgrowth of fully or partially released buds, whereas local CK enhancement can stimulate initial bud release.

SLs might affect CKs in the plant; pea and Arabidopsis SL increased-branching mutants have depleted levels of CK in the xylem sap relative to wild-type plants due to long-distance feedback regulation operating in the SL branching pathway (Beveridge et al., 1997a, 1997b; Morris et al., 2001; Foo et al., 2007). However, CK levels in the shoot of pea and Arabidopsis SL mutants are no different from those in the wild type (Foo et al., 2007). CK might also affect SL biosynthesis; *MAX4* promoter::*GUS* reporter studies in Arabidopsis suggest that CK can prevent auxin-induced up-regulation of *MAX4* SL biosynthesis gene expression in root tissue (Bainbridge et al., 2005).

TEOSINTE BRANCHED1 (*TB1*) in monocots and its homolog *BRANCHED1* (*BRC1*) in Arabidopsis and pea encodes a TCP (for TB1, CYCLOIDEA, and PCF domain) transcription factor that is expressed specifically in nongrowing inhibited axillary buds; *tb1* and *brc1* mutants have increased bud outgrowth phenotypes (Doebley et al., 1997; Takeda et al., 2003; Aguilar-Martínez et al., 2007; Finlayson, 2007; Braun et al., 2011). The TCP family of transcription factors is implicated in many different plant development and morphogenic processes (Doebley et al., 1997; Nath et al., 2003; Aguilar-Martínez et al., 2007; Schommer et al., 2008; Tatematsu et al., 2008; Pruneda-Paz et al., 2009). Several TCP family members control cell proliferation through regulation of the cell cycle, but few TCP targets have been identified (for review, see Martín-Trillo and Cubas, 2010). Being bud-specific genes necessary for the inhibition of outgrowth, *TB1* and *BRC1* are good candidates for functioning downstream of SLs, potentially functioning as integrators of

different hormonal and environmental regulatory factors.

TB1 and *BRC1* action downstream of SLs is supported by (1) the lack of bud outgrowth inhibition by SL treatment in the Arabidopsis and pea *brc1* mutants (Brewer et al., 2009; Braun et al., 2011) and the rice *Ostb1* mutant (Minakuchi et al., 2010); (2) reduced expression of *PsBRC1* and *AtBRC1* in SL mutant buds (Aguilar-Martínez et al., 2007; Finlayson, 2007; Braun et al., 2011); and (3) the promotion of *PsBRC1* expression by SL treatment in pea (Braun et al., 2011). However, in rice, no reduction in *OsTB1* expression was observed in the SL mutant buds tested, and *OsTB1* expression was not affected by SL treatment (Minakuchi et al., 2010). In pea and rice, *PsBRC1* and *OsTB1* expression is repressed by CK treatment (Minakuchi et al., 2010; Braun et al., 2011). This repression is unlikely to require SLs, as the CK response of *PsBRC1* and *OsTB1* is RMS4/D3 independent.

Exactly how SLs integrate with local and long-distance CKs to antagonistically control bud outgrowth is unclear, and the extent of their antagonism in bud outgrowth assays is untested. In this paper, we elucidate how local and long-distance xylem sap CK integrates with SLs to regulate bud outgrowth and examine the effect of these competing hormones on the expression of *PsBRC1*, supporting a model for SL and CK action at the bud via *PsBRC1*.

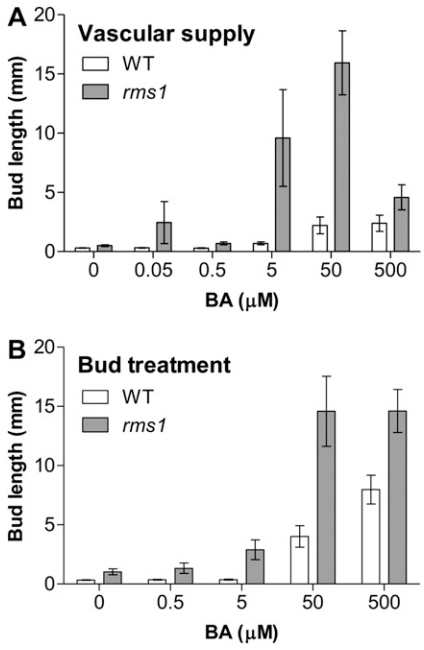

Figure 1. SL-deficient plants are more responsive to CK than wild-type (WT) plants. The synthetic CK, BA, was supplied to the stem vascular stream below node 3 (A) or applied directly to the bud at node 3 (B) of 9-d-old wild-type and *rms1-2* (Torsdag) pea plants. Bud length at node 3 was measured 7 d after treatment. Data are means ± SE (n = 7–12).

RESULTS

SL-Deficient *rms1* Buds Are More Sensitive to CK Supplied to the Vasculature Than Are Wild-Type Buds

Our previous study showed that wild-type buds are less sensitive than SL-insensitive *rms4* buds to CK supplied to the vasculature (Dun et al., 2009b). To examine the degree of this CK insensitivity of wild-type buds and to compare responses to xylem-delivered and locally supplied CKs, we performed dose-response experiments where increasing concentrations of the synthetic CK BA were supplied to the vasculature or directly to the bud of wild-type and SL mutant plants. We utilized growth conditions and a genetic background that enabled us to select a node, node 3, where the bud does not normally grow into a branch in SL mutant or wild-type pea plants.

Vascular supply of increasing doses of BA caused little increase in wild-type bud length over 7 d at node 3, the first node above the site of supply. Fifty and 500 μM BA caused a similarly small but significant increase in bud length, whereas 0.05, 0.5, and 5 μM BA caused no significant effect on bud growth (Fig. 1A). Where applied directly to wild-type buds, 50 μM BA was the lowest concentration tested that stimulated growth, and this was enhanced at 500 μM BA (Fig. 1B). *rms1* SL-deficient buds showed a greater outgrowth response to vascular supply of BA than wild-type buds, both in the magnitude of the growth response and in the sensitivity to the concentration of BA supplied (Fig. 1A). In response to vascular supply of 5 μM BA, *rms1* buds grew to a length about four times greater than that of 50 or 500 μM BA-treated wild-type buds. In response to BA applied directly, we also found that *rms1* buds responded with longer bud lengths and at lower doses than did wild-type buds (Fig. 1B). Again, *rms1* buds showed a significant growth response to direct treatment of 5 μM BA (Fig. 1B; $P < 0.01$), whereas 50 μM BA was required for this response in the wild type.

It must be noted that vascular supply of 500 μM BA, while increasing bud lengths, appeared to be toxic to the plant, causing stem thickening and a 63% and 67% reduction in overall plant height in the wild type and *rms1*, respectively, in the 7 d since treatment (data not shown). Since this highest tested dose of BA supplied to the vasculature appeared toxic and yet resulted in little outgrowth, we conclude that wild-type buds are somewhat resistant to the stimulatory effect of BA supplied to the vasculature. Additionally, although wild-type buds respond better to locally applied BA than to that supplied to the vasculature at a distance (Fig. 1), wild-type buds appeared less responsive to the stimulatory effect of BA when applied directly to the bud compared with *rms1* SL mutant buds. Therefore, we conclude that SL deficiency alleviates resistance to BA supplied locally or at a distance.

To confirm that the lack of wild-type bud response to the vascular supply of CK is not specific to the synthetic CK, BA, we compared the effect of vascular

supply of the predominant endogenous CK in pea xylem sap, trans-zeatin riboside (tZR; Beveridge et al., 1997a), with that of BA. As expected, wild-type buds were resistant to the vascular supply of both tZR and BA, while *rms1* SL-deficient buds exhibited growth responses to both forms of CK (Supplemental Fig. S1A). The highest tested dose of BA and tZR again appeared toxic to the plants, with 1,000 μM BA causing 46% and 50% reductions, and tZR causing 26% and 46% reductions, in wild-type and *rms1* plant height, respectively, in the 7 d since treatment (Supplemental Fig. S1B). As the genotypic differences were maintained between BA and tZR responses, all further experiments were conducted with BA.

SL Reduces the CK Promotion of Bud Growth

Since SL deficiency appears to lead to increased CK responses, particularly when the CK treatment method is vascular supply, the ability of the synthetic SL, GR24, to reduce CK bud growth responses was examined. Vascular supply of GR24 in combination with BA reduced the BA-induced bud growth of SL-deficient *rms1* plants, but not that of SL-insensitive *rms4* plants, at the node above vascular supply (Fig. 2A). This indicates that GR24 reduces the CK response through the RMS4 SL response pathway. As found in Figure 1 and Supplemental Figure S1A, vascular supply of BA caused little bud growth in wild-type plants; however, vascular supply of GR24 did not prevent this tiny amount of bud growth (Fig. 2A). This indicates that the buds of wild-type plants treated through the vasculature with BA are not responding in the same way as other buds, including wild-type buds treated directly with BA. Instead, the relatively small effect of vascular supply of CK in the wild type may not have been genuine bud growth.

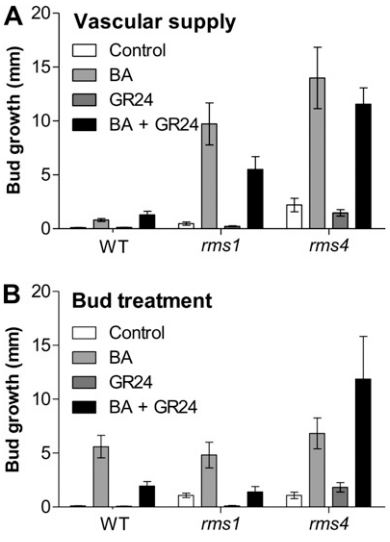

Figure 2. SL reduces the stimulatory effect of BA on bud outgrowth in an RMS4-dependent manner. The synthetic SL, GR24 (1 μM), and/or BA (50 μM) were supplied to the vasculature below node 3 (A) or applied directly to the bud at node 3 (B) of 10-d-old wild-type (WT), *rms1-2*, or *rms4-1* (Torsdag) pea plants. Bud growth at node 3 was measured 7 d after treatment. Data are means ± SE (n = 7–12).

In contrast to Figure 1B, where *rms1* SL-deficient buds showed a greater response than wild-type buds to direct treatment with BA, particularly at lower doses, on this occasion we found that wild-type and *rms1* buds responded equally well to direct treatment with a high (50 μM) dose of BA (Fig. 2B). When applied directly to wild-type and *rms1* buds, GR24 in combination with BA significantly reduced the BA-induced bud growth (Fig. 2B; $P < 0.01$). Again, as expected, bud growth in SL-insensitive *rms4* plants treated with or without BA showed no inhibition by GR24 treatment. Therefore, it appears that *RMS4*-dependent SL signaling and CK act antagonistically to regulate bud growth.

To determine if the apparent antagonistic relationship between SL and CK in the control of bud growth functions in the classical dose-dependent manner expected of hormone signaling, dose-response experiments were performed. We used *rms1* SL-deficient plants for these experiments because their buds show a growth response to vascular supply of BA and to GR24 (Gomez-Roldan et al., 2008). We again utilized the bud at node 3 that does not grow into a branch under our growth conditions unless stimulated to do so. Accordingly, in contrast to Gomez-Roldan et al. (2008), where a bud that would normally grow into a branch was examined, increasing doses of GR24 in the absence of BA had only a minor effect on *rms1* node 3 bud length (Fig. 3A). Increasing concentrations of GR24 resulted in a dose-dependent reduction of BA-induced bud growth (Fig. 3A). Likewise, increasing concentrations of BA supplied to the vasculature decreased the bud-inhibiting effectiveness of GR24 (Fig. 3B). Therefore, it appears that GR24 and BA act antagonistically in a dose-dependent manner on bud growth when both are supplied to the vasculature.

Since buds of wild-type plants (which have endogenous SLs) did not respond much to the vascular supply of BA in comparison with buds of SL-deficient or -insensitive mutant plants, and vascular supply of the synthetic SL, GR24, reduced BA-induced bud growth in SL-deficient plants, we hypothesized that systemic SLs might influence the delivery of CKs, including BA, to axillary buds. We also considered that the converse hypothesis may be true: CKs, including BA, might affect the delivery of SLs, including GR24, to axillary buds. To test these hypotheses, we determined (1) if vascular supply of GR24 could reduce BA-induced outgrowth when BA was applied directly to the axillary bud and (2) if GR24 applied directly to the axillary bud could reduce BA-induced growth when BA was supplied to the vasculature. If either hormone acts by preventing the delivery of the other from the bud to the stem, it would be expected that in order to do so, it would need to function in the stem and not solely in the bud itself. These experiments were con-

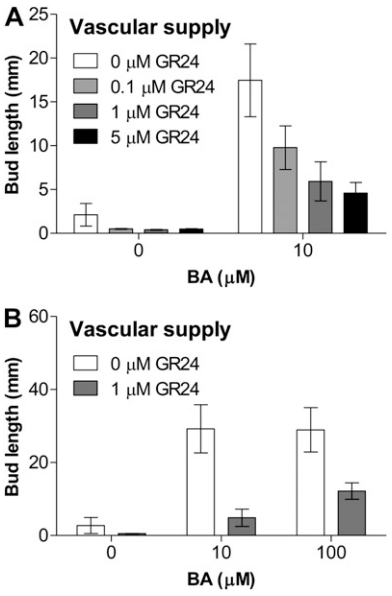

Figure 3. SL reduces the stimulatory effect of CK on bud outgrowth in SL-deficient plants in a dose-dependent manner. GR24 and/or BA were supplied to the vasculature below node 3 of 9-d-old (A) and 10-d-old (B) *rms1-2* (Torsdag) pea plants. Bud length at node 3 was measured 7 d after treatment. Data are means ± SE (n = 14 [A] and 9–12 [B]).

ducted using the *rms1* SL-deficient mutant, so that the exogenously supplied GR24 would be the only SL available to the plant. We were not able, however, to create the converse situation, where the exogenously supplied BA was the only CK available.

We found that GR24 supplied to the vasculature significantly reduced bud growth stimulated by BA application to the bud (Fig. 4A; $P < 0.01$) and that GR24 applied to the bud was effective at inhibiting growth induced by the vascular supply of BA (Fig. 4B; $P < 0.01$). While it is possible that the vascular supply of GR24 limited the endogenous supply of CK to the bud, this does not account for the significant reduction in BA-induced growth observed after vascular supply of GR24 (Fig. 4A). It is unlikely, therefore, that SLs act primarily to inhibit bud outgrowth by affecting CK delivery to buds. Similarly, it is unlikely that CKs promote bud outgrowth by affecting SL delivery to buds.

SL and CK Act Antagonistically on the Same Gene Target, *PsBRC1*

The physiological data presented here (Figs. 1–4; Supplemental Fig. S1A) support the notion that SL and CK act antagonistically on bud outgrowth control, potentially acting on a common target, possibly at the bud. It was recently reported that the expression of the TCP transcription factor already implicated in bud outgrowth control, *PsBRC1*, is up-regulated by GR24 and down-regulated by BA in pea axillary buds (Braun et al., 2011). Here, we show that the expression of *PsBRC1* in buds treated with BA, GR24, or BA and GR24 (Fig. 5) is negatively correlated with bud growth (Fig. 2B). As seen in Braun et al. (2011), the expression of *PsBRC1* was decreased in SL mutant buds compared with wild-type buds, addition of BA reduced the expression of *PsBRC1* in buds of wild-type and SL mutant plants, and addition of GR24 increased the expression of *PsBRC1* in buds in an *RMS4*-dependent manner (Fig. 5). Combined treatment of BA and GR24 resulted in an intermediate level of *PsBRC1* expression in wild-type and *rms1* buds (Fig. 5), which correlates nicely with the intermediate growth responses observed for this treatment combination (Fig. 2B). These data support the premise that SLs and CK act antagonistically on bud outgrowth control by regulating the expression of *PsBRC1* in axillary buds.

To determine if BA or GR24 regulates *PsBRC1* expression directly without the need for de novo protein synthesis, we tested the ability of BA or GR24 to regulate the expression of *PsBRC1* in the presence of the protein synthesis inhibitor cycloheximide (CHX; Koshiba et al., 1995; Arite et al., 2007). Treatment of wild-type and *rms1* SL-deficient buds with CHX alone caused 77% and 69% increases, respectively, in *PsBRC1* transcript abundance (Fig. 6), indicating that the transcription of *PsBRC1* is normally repressed, or that the *PsBRC1* transcript is normally destabilized, by a rapidly turned over protein. The reduction of protein synthesis by CHX treatment did not prevent BA from

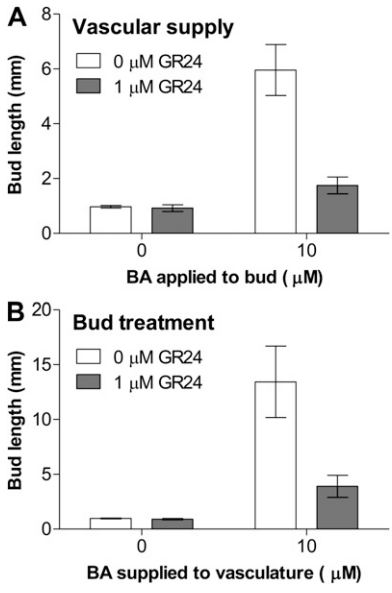

Figure 4. SL reduces CK-induced outgrowth whether supplied locally or to the vasculature of SL-deficient plants. A, Zero or 1 μM GR24 was supplied to the vasculature below node 3, while 0 or 10 μM BA was supplied directly to the bud at node 3, of 10-d-old *rms1-2* (Torsdag) pea plants. B, Zero or 1 μM GR24 was supplied to the bud at node 3, while 0 or 10 μM BA was supplied to the vasculature below node 3, of 10-d-old *rms1-2* (Torsdag) pea plants. Bud length at node 3 was measured 7 d after treatment. Data are means ± SE (n = 14).

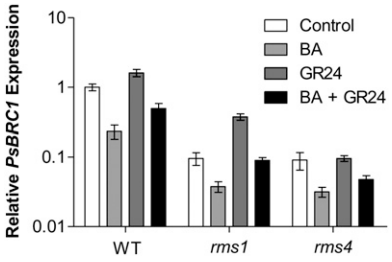

Figure 5. SL and CK act antagonistically on the same target gene, *PsBRC1*. The bud at node 3 of 8-d-old wild-type (WT), *rms1-2*, or *rms4-1* (Torsdag) pea plants was treated for 6 h with or without GR24 (1 μM) and/or BA (50 μM). Expression of *PsBRC1* in the bud at node 3 is represented relative to the wild-type control; *EF1α* was used as the internal reference gene. Data are means ± SE ($n = 3$ pools of 30 plants).

reducing the expression of *PsBRC1* (Fig. 6A), indicating that the synthesis of a rapidly turned over protein is not required for BA to exert its effect. Likewise, CHX treatment did not prevent the synthetic SL, GR24, from increasing the expression of *PsBRC1* (Fig. 6B). These data suggest that CK and SL may interact quite closely with *PsBRC1* or with stable proteins that, in turn, interact directly with *PsBRC1* to control bud outgrowth in pea.

Physiological experiments show that although buds of wild-type plants are somewhat resistant to stimulation by CK supplied to the vasculature relative to buds of SL mutants, they do exhibit a small increase in length in response to CK supplied to the vasculature (Figs. 1A and 2A; Supplemental Fig. S1A). To determine if this small increase in bud length or lack of sustained bud growth in the wild type is due to *PsBRC1* not being regulated by vascularly derived BA, *PsBRC1* expression was examined in wild-type buds 24 h after BA treatment either directly to the bud or to the vasculature (Fig. 7). Supplying BA to the vasculature or to the buds directly caused comparable reductions in the expression of *PsBRC1* in the bud (Fig. 7). Markers for bud dormancy, *PsDRM1*, *PsDRM2* (Stafstrom et al., 1998), and *PsAD1* (Madoka and Mori, 2000), also showed similar responses to BA, whether supplied directly to the bud or to the vascular stream. Within the same 24-h time frame, comparable buds treated with BA, directly or supplied to the vasculature, showed the same increase in bud length. However, as found previously (Figs. 1A and 2A; Supplemental Fig. S1A), wild-type buds did not show continued growth when BA was supplied to the vasculature compared with direct treatment (data not shown). These data suggest that the difference in the longer term growth response between wild-type buds treated directly with BA and those treated from a distance is not due to a failure to initiate molecular responses in the bud. They also suggest that CK supplied to the vasculature does indeed make it to the bud and that the small increase in wild-type bud size after BA is supplied to the vasculature (Figs. 1A and 2A; Supplemental Fig. S1A) is genuine growth.

CK Biosynthesis Genes Are Up-Regulated in SL Mutants

SL-deficient and response mutants in pea (and other species) have depleted CK levels in the xylem sap derived from the roots (Beveridge et al., 1997a, 1997b, 2009; Foo et al., 2007; Waldie et al., 2010) yet normal levels of CKs in shoot tissue (Foo et al., 2007). To examine the possibility that SLs might regulate bud outgrowth by affecting the expression of CK biosynthesis genes, *PsIPT1* and *PsIPT2* expression was observed in tissue containing internode and node 3 (including bud) from wild-type, *rms1* SL-deficient mutant, and *rms4* SL response mutant plants (Fig. 8). Interestingly, the expression of *PsIPT1*, but not *PsIPT2*, was increased in *rms1* and *rms4* SL mutants compared with wild-type plants, consistent with the possibility that SLs negatively regulate the expression of CK biosynthesis genes in the shoot.

CK Biosynthesis Genes Are Not Affected by GR24 in Wild-Type Isolated Segments

To determine if the increase in *PsIPT1* CK biosynthesis gene expression observed in the pea SL mutants (Fig. 8) is directly due to the loss of SL response, we tested if GR24 can regulate the expression of *PsIPT1* or *PsIPT2* within a short time frame by incubating isolated segments from internode 4 of young wild-type pea seedlings with or without 1 μM GR24 and/or 10 μM IAA for 4 h (Fig. 9). Auxin (IAA) was used as a control, as it is known to reduce the expression of *PsIPT1* and *PsIPT2* in intact, decapitated, and in vitro studies (Tanaka et al., 2006). As expected, the expression of *PsIPT1* and *PsIPT2*

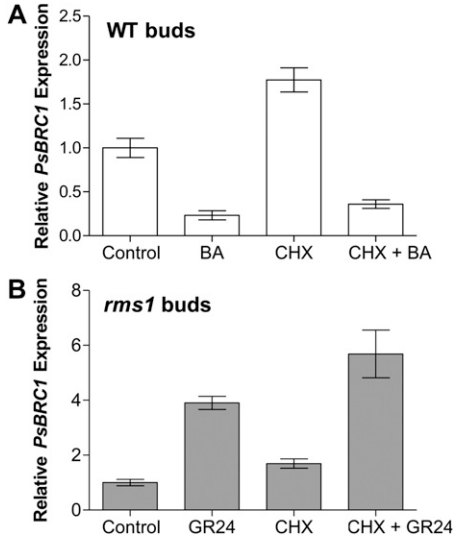

Figure 6. SL and CK regulate *PsBRC1* without the need for de novo protein synthesis. The bud at node 3 of 8-d-old wild-type (WT; A) and *rms1-2* (Torsdag; B) pea plants was treated for 6 h with or without GR24 (1 μM), BA (50 μM), and/or CHX (10 μM). Expression of *PsBRC1* in the bud at node 3 is represented relative to controls; *EF1α* was used as the internal reference gene. Data are means ± SE ($n = 3$ pools of 30 plants) and are from the same experiment as in Figure 5.

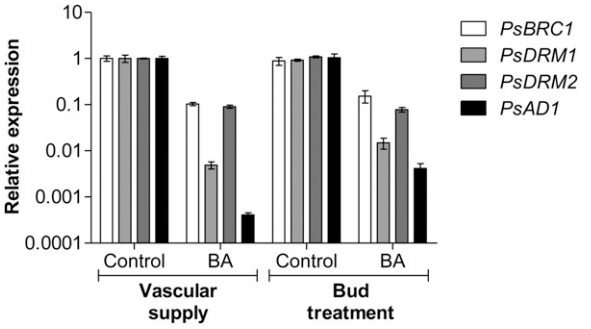

Figure 7. CK supplied locally or from a distance decreases the expression of *PsBRC1* and bud dormancy markers 24 h after treatment. Zero or 50 μM BA was supplied to the vasculature below node 3 or applied directly to the bud at node 3 of 9-d-old wild-type Torsdag pea plants. Expression of *PsBRC1*, *PsDRM1*, *PsDRM2*, and *PsAD1* in the bud at node 3 24 h after treatment is represented relative to the vascular supply control treatment; *EF1α* was used as the internal reference gene. Data are means ± SE (n = 3 pools of 28–30 plants).

was increased in isolated segments incubated in control buffer compared with that observed in intact plants, as a segment has no apical source of auxin (Fig. 9). Incubation with IAA reduced the expression of *PsIPT1* and *PsIPT2* in segments relative to incubation with control solution, returning expression to levels similar to those observed in comparable intact plants. With or without IAA, the synthetic SL, GR24, had no effect within the 4-h time frame on the expression of *PsIPT1* or *PsIPT2*. Therefore, we conclude that any regulation of *PsIPT* expression by SL is either indirect (i.e. takes longer than 4 h) or is specific to node or bud tissues, which were not tested here.

DISCUSSION

SL Mutants Show an Increased Response to Local and Long-Distance Vascularly Supplied CK

By using buds on SL mutant plants that do not normally grow into a branch under certain conditions, we were able to directly compare the responses of wild-type and SL mutant plants to CK. SL mutant buds showed increased bud growth responses to CK (BA or tZR) treatments than wild-type buds when CK was supplied to the stem (Figs. 1A and 2; Supplemental Fig. S1A). At the node above the site of supply, wild-type buds showed a poor response to CK (BA and tZR) supplied to the vasculature, increasing to only a few millimeters in length. It is unlikely that higher concentrations of CK supplied to the stem would induce increased outgrowth of wild-type buds, as the highest tested concentrations of CK appeared toxic to the plant, leading to stem thickening and reduced overall plant height (Supplemental Fig. S1B). Wild-type buds, however, did grow in response to BA supplied locally to the bud (Figs. 1B and 2B), but SL mutant buds showed increased bud growth responses at lower concentrations of applied BA (Fig. 1B).

The increased-branching phenotypes of CK-overproducing plants, for example *altered meristem program1* (Chaudhury et al., 1993), are likely due to increases in CK local to the bud. Other studies have found that long-distance supply of CK from rootstocks of CK-overproducing 35S-*ipt* transgenic lines is not able to increase branching in nontransgenic wild-type shoots (Faiss et al., 1997). This is consistent with our finding that wild-type buds show a poor response to vascularly supplied CK. However, our results with SL mutants show that CKs delivered in the vasculature over a long distance can enhance branching. The distinction between local and long-distance CK and the differences between wild-type and SL mutants in CK response need to be explored in the future. Our results here support the premise that long-distance CK requires buds to be released from SL inhibition in order to induce branch growth (Dun et al., 2009b).

It is unclear how buds distinguish between vascular and local supply of CK. It could be that vascular supply of CK to wild-type plants results in less delivery of CK to the bud compared with that in SL mutants, due to some indirect effect such as a difference in water supply to, or transpiration of, wild-type and mutant buds; indeed, nongrowing SL mutant buds were larger than corresponding nongrowing wild-type buds (see size of control-treated buds in Figs. 1 and 2 and Supplemental Fig. S1A). However, this suggestion is not supported by the dose-response experiment, where a 10-fold difference in vascular CK supply caused no enhancement of growth beyond the lowest dose that caused a small growth response (Fig. 1A). Alternatively, it could be that SL mutant buds are at a more responsive stage than wild-type buds due to a more direct consequence of reduced SL signaling (Dun et al., 2006). Grafting experiments in pea suggest that long-distance supply of CK in the xylem is only effective at inducing the branch growth of buds that are already released from inhibition by SL (Dun et al., 2009b).

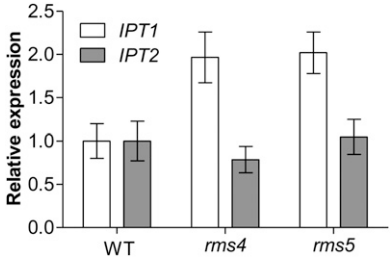

Figure 8. *PsIPT1* but not *PsIPT2* expression is increased in SL mutant stem tissue. Internode 3 and node 3 (including bud) were harvested from 12-d-old (four to five leaves expanded) wild-type (WT), *rms4-1*, and *rms5-3* (Torsdag) pea plants. Expression is represented relative to the wild type; *18S* was used as the internal reference gene. Data are means ± SE (n = 7–8).

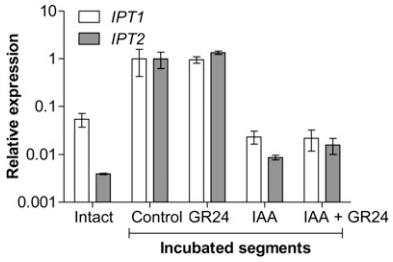

Figure 9. GR24 does not affect *PsIPT1* or *PsIPT2* expression in isolated wild-type stem tissue segments within 4 h. Twelve-millimeter segments from internode 4 of 12-d-old wild-type Torsdag pea plants were incubated for 4 h with 0 or 1 μM GR24 or with 0 or 10 μM IAA; equivalent internode tissue was also harvested from intact plants as another control (Intact). Expression is represented relative to the incubated segment control; *18S* was used as the internal reference gene. Data are means ± SE (n = 3 pools of seven plants).

Interactions between SL and CK Occur at the Bud

Using physiological approaches, we show that GR24 and BA do not affect each other's delivery to the shoot to control bud outgrowth (Fig. 4). When BA was supplied to the vasculature, GR24 was effective at reducing BA-induced bud growth whether supplied to the vasculature or to the bud (Figs. 2A, 3, and 4). Similarly, when the bud was treated directly with BA, GR24 reduced the BA-induced bud growth whether supplied to the vasculature or to the bud (Figs. 2B and 4). These results suggest that the presence of SL in the main stem transport pathway is not integral to its function; SL can function at the bud to inhibit CK-induced bud outgrowth. This is consistent with the idea that *PsBRC1* expression in axillary buds is a target of SL and CK to achieve bud outgrowth regulation.

There is evidence to suggest that SLs might affect CK levels. SL-deficient and response mutants in pea and Arabidopsis have decreased levels of CK in the xylem sap derived from the roots (Beveridge et al., 1997a, 1997b, 2009; Foo et al., 2007; Waldie et al., 2010). However, levels of CK are normal in shoot tissue of pea and Arabidopsis SL mutants (Foo et al., 2007). This differs from rice *D10*-RNA interference (RNAi) plant lines (presumed SL deficient) that had increased levels of CK in nodal tissue adjacent to branches that grew longer in *D10*-RNAi plants than in the wild type (Zhang et al., 2010). However, *IPT8* was the only CK biosynthesis gene tested to be elevated in expression in the rice *D10*-RNAi plant lines in comparison with wild-type controls at a node where the branches grew (Zhang et al., 2010). Here, we show that despite normal levels of CK in the shoot tissue (Foo et al., 2007), the expression of *PsIPT1*, but not *PsIPT2*, is elevated in SL mutants (Fig. 8). This increased *PsIPT1* gene expression could correspond to increased CK biosynthesis in the shoot, perhaps as a mechanism to compensate for the reduced contribution of CK from the roots of SL mutants. Indeed, pea SL mutants have normal levels of CK in the shoot. However, the increased expression of *PsIPT1* is likely a long-term consequence of SL deficiency/insensitivity rather than a direct effect, as GR24 had no effect of *PsIPT1* expression within 4 h in an isolated stem segment assay (Fig. 9). It must be noted that the effect of GR24 on *PsIPT1* expression was only tested in an in vitro system using wild-type segments, which should be SL deficient prior to GR24 treatment, due to the removal of the apical supply of auxin (Foo et al., 2005), and which may behave differently to intact plants (Dun et al., 2006). Nevertheless, even in auxin-treated segments, where *PsIPT* gene expression was restored to near intact levels, no effect of GR24 on *PsIPT* gene expression was observed.

Although not tested here, it is also possible that CK might affect SL biosynthesis. Studies in Arabidopsis showed that BA can prevent the auxin-induced up-regulation of GUS activity in the root of *MAX4* promoter::*GUS* transgenic plants (Bainbridge et al., 2005). In any case, any effects of SL on CK levels or CK on SL levels are unlikely to be their primary function in bud outgrowth control. Rather, the physiological data indicate increased CK sensitivity in SL mutants, and as discussed below, that GR24 can decrease, and BA can increase, the expression of *PsBRC1* in the bud (Fig. 5).

PsBRC1 Is Regulated by SL and CK

Here, we show that the SL and CK bud outgrowth regulatory pathways function antagonistically, potentially converging on a common target transcription factor, *PsBRC1*, previously shown to function in bud outgrowth control at the bud. At the physiological level, we show that the antagonistic action of SL and CK can occur directly in buds and that an antagonistic interaction between these hormones does not, in itself, require the vascular stream transport pathway. At the molecular level, we show that this antagonism is likely achieved, at least in part, through the regulation of *PsBRC1* expression in the bud.

Several observations support the premise that *TB1* and *BRC1* play an important and integral role in bud outgrowth regulation, acting specifically at the axillary bud. Expression levels of *TB1*, a monocot homolog of *BRC1*, were previously demonstrated to underlie differences in branching between maize (*Zea mays mays*) and its ancient progenitor teosinte (*Zea mays parviglumis*; Doebley et al., 1997). Additionally, *tb1* and *brc1* mutants in various species have increased-branching phenotypes, and *TB1* and *BRC1* are expressed predominantly in axillary buds (Takeda et al., 2003; Aguilar-Martínez et al., 2007; Finlayson, 2007; Minakuchi et al., 2010; Braun et al., 2011).

The expression of *TB1* and *BRC1* appears to be under close hormonal control. *BRC1* expression is reduced in buds of Arabidopsis and pea SL mutant plants (Fig. 5; Aguilar-Martínez et al., 2007; Finlayson, 2007; Braun et al., 2011), and *BRC1* expression in pea is increased by exogenous treatment with the synthetic SL, GR24

(Figs. 5 and 6B; Braun et al., 2011). Additionally, where tested, treatments that promote bud outgrowth, such as decapitation, which affects the levels of several plant hormones, or CK treatment, decrease *TB1* or *BRC1* expression in rice, pea, and Arabidopsis (Figs. 5, 6A, and 7; Aguilar-Martínez et al., 2007; Minakuchi et al., 2010; Braun et al., 2011).

We have further dissected the integration of SL and CK signaling by BRC1 by examining the effects of single and joint applications of these hormones on bud outgrowth and gene expression and by determining whether each hormone acts directly or indirectly in regulating *PsBRC1* expression. We found that the expression of *PsBRC1* in axillary buds of pea a short time (6 h) after direct treatment with BA and/or GR24 correlates with final bud outgrowth phenotypes, including the intermediate outgrowth response observed when the hormones are applied together (Figs. 2B and 5). This supports the premise that SL and CK regulate bud outgrowth via the regulation of this gene in the bud.

We also found that the expression of *PsBRC1* and bud dormancy markers in buds of wild-type plants treated with BA to the vasculature or bud correlates with the current bud growth status at the time of harvest (Fig. 7). However, further research is required to determine why buds of wild-type plants supplied with BA to the vasculature do not continue growth into a branch.

By treatment with the translation inhibitor CHX (Koshiba et al., 1995; Arite et al., 2007), we show that GR24 and BA regulation of *PsBRC1* expression does not require protein synthesis (Fig. 6B). This finding also indicates that SL and CK probably do not interact to regulate bud outgrowth by modulating the level or transport of one or the other hormone or some other hormone or process upstream of *PsBRC1*.

BRC1 Provides an Effective Control System in Buds

The integration of different hormonal signals by a single protein, BRC1, provides an effective control system for bud outgrowth. We propose that SL mutant buds show increased response to CK due to their already decreased expression of *PsBRC1* (Fig. 5). This decreased expression of *PsBRC1* relative to the wild type would essentially prime the bud for an enhanced response to CK while not yet being sufficient to fully induce a bud outgrowth response. Indeed, the "non-growing" SL mutant buds used in this study were slightly larger than the corresponding wild-type buds (Figs. 1 and 2; Supplemental Fig. S1). Therefore, we expect that there is a certain level or threshold below which *PsBRC1* expression must drop before bud outgrowth ensues. This would in part enable the quantitative integration of multiple factors, hormonal, developmental, and environmental, that affect bud outgrowth (Kebrom et al., 2006, 2010; Aguilar-Martínez et al., 2007; Finlayson, 2007; Minakuchi et al., 2010; Braun et al., 2011).

Exactly how *BRC1* and *TB1* function as integrators of many signals controlling bud outgrowth is not yet understood. Being members of the TCP protein family, *BRC1* and *TB1* possess a basic helix-loop-helix motif that facilitates DNA binding and likely function as transcriptional activators or repressors (for review, see Martín-Trillo and Cubas, 2010). Some TCPs in the same class as *BRC1* and *TB1* are involved in the regulation of cell cycle progression (Masuda et al., 2008), while others might regulate the transcription of cell cycle marker genes (Nath et al., 2003; for review, see Martín-Trillo and Cubas, 2010). In contrast, a TCP family member has also been potentially implicated in a nontranscriptional control process involving protein-protein interactions (Suzuki et al., 2001). Further studies are required to elucidate how the modulation of *BRC1* and *TB1* expression translates to a modulation of axillary bud cell proliferation and growth. However, it is tempting to speculate that the antagonistic SL and CK signals are integrated in the bud by BRC1, which simply suppresses cell cycle progression and hence cell division and development.

Impact of Theories on Shoot Branching

The antagonism between SL and CK in bud outgrowth control might explain conflicting findings with regard to the SL inhibition of bud outgrowth after decapitation or segment isolation and that relate to two predominant hypotheses on SL function. Bennett et al. (2006), Crawford et al. (2010), and Prusinkiewicz et al. (2009) propose that SL functions in the main stem to reduce auxin transport, which reduces auxin canalization out of axillary buds (for review, see Domagalska and Leyser, 2011). Alternatively, although not necessarily exclusively, Brewer et al. (2009) suggest that SLs may act directly in axillary buds. This latter hypothesis is supported here by the characterization of a bud-specific gene, *BRC1*, that is essential for bud growth suppression and that is tightly regulated at the level of gene expression by SL and CK. In addition to the bud-specific expression of *BRC1*, the SL and CK interaction in buds is supported by physiological evidence that vascularly supplied hormones interact equally well with locally supplied or vascularly supplied hormones to antagonistically influence bud outgrowth (Figs. 3 and 4) and that both local and vascularly supplied CK can affect *PsBRC1* expression (Fig. 7).

Using this second hypothesis, we can reinterpret findings on SL responses in isolated stem segments and decapitated plants. In both circumstances, auxin levels are depleted in the stem adjacent to the axillary bud; hence, CK levels must be substantially elevated (Shimizu-Sato et al., 2009). Consequently, in both the isolated nodal stem segment and decapitated experimental systems, both auxin and CK levels are affected, albeit presumably to different magnitudes. Moreover, even in the wild type, auxin depletion will also lead to SL depletion (Foo et al., 2005; Hayward et al., 2009). In

Arabidopsis and chrysanthemum isolated nodal stem segment assays, a supply of auxin is necessary for the repression of bud growth by basal supply of SL in the medium (Crawford et al., 2010; Liang et al., 2010), supporting the auxin transport model. By contrast, SL treatment directly to the uppermost bud of decapitated pea plants prevents decapitation-induced growth in the absence of an apical auxin supply (Brewer et al., 2009), supporting the direct SL model. Restoring auxin to the system will also reduce CK levels. Consequently, in view of our data on the quantitative effects of CK on SL responses, the different responses in the different systems may indeed relate to different CK levels and, hence, different responses to SL. Further studies need to explore the CK content in these different systems to gauge the impact of SL responses.

In the direct action model, where SL acts directly in buds via *BRC1*, it may do so without requiring a change in auxin status in the stem and yet may also repress auxin canalization from buds (Brewer et al., 2009). Nevertheless, there are several reports of SL affecting the localization of PIN auxin efflux carriers or auxin transport in stems (Bennett et al., 2006; Lazar and Goodman, 2006; Lin et al., 2009; Crawford et al., 2010). Given that SL function is now emerging as important in other developmental processes, such as root development, it is possible that its effect on auxin transport may be greater for those processes rather than for shoot branching.

CONCLUSION

We show that the expression of *PsBRC1* is tightly regulated by SL and CK and that the level of expression correlates with bud outgrowth responses. The results presented here support a model in which SL up-regulates the expression of *PsBRC1* while local CK represses its expression; the expression of *PsBRC1* in the bud represses the development of the axillary bud into a growing branch. In addition, long-distance supply of CK enhances the development of an axillary bud into a branch.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Measurements

Unless otherwise stated, pea (*Pisum sativum*) plants were grown two per 2-L pot containing potting mix (Green Fingers B2 Potting Mix; www.greenfingerspottingmix.com) with approximately 2 g of Osmocote (Scotts); Flowfeed EX7 liquid nutrients (Grow Force) were supplied weekly. For the gene expression studies in Figures 5, 6, 7, and 9, plants were grown at six, six, three, and three plants, respectively, per 2-L pot filled with Green Fingers EcoZ Plus Potting Mix without Osmocote. Plants were grown in 18-h-photoperiod glasshouse conditions, as described by Ferguson and Beveridge (2009), except for Figures 5 and 6, where plants were grown under a natural photoperiod. For all experiments, the wild-type cv Torsdag (L107) was used, and the mutant lines introduced into Torsdag were *rms1-2* (*rms1-2T*), *rms4-1* (K164), and *rms5-3* (BL298).

Nodes were counted from the cotyledonary node as node 0. Bud and branch lengths were measured with digital calipers. Plant height was measured from node 1 to the shoot apex.

For statistical analyses, one-way ANOVA with Tukey's posttest and Student's *t* test were performed using GraphPad Prism version 5.01 (GraphPad Software; www.graphpad.com).

Hormone and Chemical Treatments

BA, tZR, and GR24 were supplied in a volume of 1.5 mL to the main stem vasculature below node 3 or applied in a volume of 10 μL directly to the bud at node 3, as described by Gomez-Roldan et al. (2008) and Dun et al. (2009b), respectively, except for solutions that contained 0.09% dimethyl sulfoxide for the experiment including tZR treatments (Supplemental Fig. S1). For Figures 5, 6, and 7, treatments, including those with CHX, were applied to the bud at node 3 as described above, but solutions also contained 0.1% dimethyl sulfoxide.

In Vitro Hormone Treatments

Individual biological replicates consisting of seven 12-mm segments of stem tissue from internode 4 were incubated in 9 mL of incubation buffer with gentle shaking in the light for 4 h. For hormone treatments, incubation buffer (Theologis et al., 1985) was supplemented with GR24 or IAA, and all treatments contained 0.01% acetone and 0.01% ethanol.

Gene Expression Analyses

Stem tissue was frozen and total RNA was isolated and quantified as described by Dun et al. (2009b; Figs. 8 and 9) and bud tissue as described by Braun et al. (2011; Figs. 5–7). cDNA synthesis and real-time PCR gene expression analyses were performed as described by Dun et al. (2009b).

Primer sequences were as follows: *PsIPT1* forward (5′-ACCGTCTTGATGCTACGGAGGTTGTGC-3′) and *PsIPT1* reverse (5′-TCTAATGGGTTACCCCTGCCACAGACG-3′; Tanaka et al., 2006); *PsIPT2* forward (5′-TGGCAGCAACATCATCCTCTGCCTGC-3′) and *PsIPT2* reverse (5′-ACCTGTGGCCCCCATTATCACTAC-3′; Tanaka et al., 2006); *PsBRC1* forward (5′-TCGAAAGACGGAATCAAACA-3′) and *PsBRC1* reverse (5′-TCCCTTGCTCTTTCTCTTGC-3′); *PsDRM1* forward (5′-CCCTCAAAGATGATGGAGCGAG-3′) and *PsDRM1* reverse (5′-CAACTTTACGCGCCGATGAAG-3′); *PsDRM2* forward (5′-TGCTCATTCTTGGCCTATTG-3′) and *PsDRM2* reverse (5′-CCACCACCAGTCCATAAC-3′); *PsAD1* forward (5′-GGTGGTGGTGGTGGTTCAATG-3′) and *PsAD1* reverse (5′-TCCGCAGCATGAAGATCACTG-3′); *18S* forward (5′-ACGTCCCTGCCCTTTGTACA-3′) and *18S* reverse (5′-CACTTCACCGGACCATTCAAT-3′; Ozga et al., 2003); *EF1α* forward (5′-TGTGCCAGTGGGACGTGTTG-3′) and *EF1α* reverse (5′-CTCGTGGTGCATCTCAACGG-3′). In all cases, error bars represent biological SE.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effects of vascular supplied cytokinins on bud growth and stem height.

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