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Strigolactone, cytokinin and bud outgrowth control

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Antagonistic action of strigolactone and cytokinin in bud outgrowth control

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ABSTRACT

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 co-ordinately regulate bud outgrowth in Pisum sativum (pea). 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. Expression of PsBRC1, 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 which 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 a hormone involved in shoot branching, certain properties of SLs were characterised based on studies of the long-distance branch-inhibiting signal in a series of increased branching mutants. These mutants include *ramosus* (*rms*) in *Pisum sativum* (pea), *more axillary growth* (*max*) in *Arabidopsis thaliana*, *decreased apical dominance* (*dad*) in *petunia hybrida* and *dwarf* (*d*) and *high tillering dwarf* (*htd*) in *Oryza sativa* (rice) (reviewed in Dun et al., 2009a; Beveridge and Kyozuka, 2010; Domagalska and Leyser, 2011). Grafting studies demonstrated that the branch-inhibiting signal can be synthesised in root or shoot tissue, moves upwards to inhibit bud outgrowth, and that a subset of the branching mutants are unable to synthesise 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; Napoli, 1996; Beveridge et al., 1997a; Morris et al., 2001; Turnbull et al., 2002; Sorefan et al., 2003; Booker et al., 2005; Simons et al., 2007). These studies also indicated 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 media 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 DIOXYGENASE (CCD) 7 and CCD8, encoded by *RMS5/MAX3/DAD3/HTD1/D17* and *RMS1/MAX4/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 *MAXI*, 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; Thimann and Skoog, 1934). However, since auxin moves strictly downwards in the polar auxin transport stream, and does not enter the axillary buds which it inhibits, second messengers for auxin were proposed (reviewed in Dun et al., 2009a; Leyser, 2009). Prior to studies with the SL increased branching mutants, CK was a candidate antagonistic second messenger for auxin (reviewed in Cline, 1991). Evidence for this includes that auxin negatively regulates expression of CK biosynthesis genes (adenosine phosphate-isopentyltransferase (IPT) genes) and positively regulates 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 SL mutants demonstrated that the branch-inhibiting signal was 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, 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 Dendranthema grandiflorum (chrysanthemum) 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 regulation of local CK biosynthesis may be an important regulator of bud outgrowth (e.g. 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 modulation of bud outgrowth remain unclear. Some studies suggest that only local CK promotes bud outgrowth (e.g. 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 (WT)
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 WT Arabidopsis buds on isolated nodal segments in split plate assays is accelerated by basal treatment with 6-benzylaminopurine (BA; synthetic CK) to the media (Chatfield et al., 2000). Consequently, our hypothesis is that xylem-sap CK may stimulate the continued outgrowth of fully or partially released buds, where as 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 WT plants due to long-distance feedback regulation operating in the SL branching pathway (Beveridge et al., 1997a; Beveridge et al., 1997b; Morris et al., 2001; Foo et al., 2007). However, CK levels in the shoot of pea and Arabidopsis SL mutants are no different to that in WT (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 (TB1, CYCLOIDEA, PCF domain) transcription factor that is expressed specifically in non-growing inhibited axillary buds; tb1 and brc1 mutants have an increased bud outgrowth phenotype (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 are implicated in many different plant development and morphogenic processes (e.g. 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 inhibition of outgrowth, TB1 and BRC1 are good candidates for functioning downstream of SLs, potentially functioning as an integrator of different hormonal and environmental regulatory factors.

TB1 and BRC1 function downstream of SLs is supported by (1) the lack of bud outgrowth inhibition by SL treatment to 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 mutants (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, Minakuchi et al. (2010) saw no reduction in OsTB1 expression 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 expression of *PsBRC1*, supporting a model for SL and CK action at the bud via *PsBRC1*.

**RESULTS**

**SL deficient rms1 buds are more sensitive to CK supplied to the vasculature than WT buds**

Our previous study showed that WT 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 WT buds and to compare responses to xylem delivered and locally supplied CKs, we performed dose-response experiments where increasing concentrations of the synthetic CK 6-benzylaminopurine (BA) were supplied to the vasculature or directly to the bud of WT and SL mutant plants. We utilised 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 WT pea plants.

Vascular supply of increasing doses of BA caused little increase in WT bud length over 7 d at node 3, the first node above the site of supply. 50 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 (Figure 1A). Where applied directly to WT buds, 50 μM BA was the lowest concentration tested that stimulated growth and this was enhanced at 500 μM BA (Figure 1B). *rms1* SL deficient buds showed a greater outgrowth response to vascular supply of BA than WT buds, both in the magnitude of growth response and in the sensitivity to concentration of BA supplied (Figure 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 WT buds. In response to BA applied directly, we also found that *rms1* buds responded with longer bud lengths and at lower doses of than did WT buds (Figure 1B). Again, *rms1* buds showed a significant growth response to direct treatment of 5 μM BA (Figure 1B; *p*<0.01) whereas a 50 μM BA was required for this response in WT.

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 WT and *rms1*, respectively, in the 7 d since treatment. Since this highest-tested dose of BA supplied to the vasculature appeared toxic and yet resulted in little outgrowth, we conclude that WT buds are somewhat resistant to the stimulatory effect of BA supplied to the vasculature. Additionally, although WT buds respond better to locally applied BA than that supplied to the vasculature at a distance (Figure 1), WT buds appeared less...
responsive to the stimulatory effect of BA when applied directly to the bud compared to \textit{rms1} SL mutant buds. We therefore conclude that SL deficiency alleviates resistance to BA supplied locally or at a distance.

To confirm that the lack of WT bud response to 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, \textit{trans} zeatin riboside (tZR; Beveridge et al., 1997b) to that of BA. As expected, WT buds were resistant to vascular supply of both tZR and BA, while \textit{rms1} SL deficient buds exhibited growth responses to both forms of CK (Figure S1A). The highest tested dose of BA and tZR again appeared toxic to the plants, with 1000 \( \mu \)M BA causing 46 % and 50 % reductions, and tZR causing 26% and 46% reductions, in WT and \textit{rms1} plant height (Figure S1B), respectively, in the 7 d since treatment. As the genotypic differences were maintained between BA and tZR responses, all further experiments were conducted with BA.

\textbf{SL reduces 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 \textit{rms1} plants, but not of SL insensitive \textit{rms4} plants, at the node above vascular supply (Figure 2A). This indicates that GR24 reduces CK response through the RMS4 SL response pathway. As in Figures 1 and S1A, vascular supply of BA caused little bud growth in WT plants; however, vascular supply of GR24 did not prevent this tiny amount of bud growth (Figure 2A). This indicates that the buds of WT plants treated through the vasculature with BA are not responding in the same way as other buds, including WT buds treated directly with BA. Instead, the relatively small effect of vascular supplied CK in WT may not have been genuine bud growth.

In contrast to Figure 1B where \textit{rms1} SL deficient buds showed a greater response than WT buds to direct treatment with BA particularly at lower doses, on this occasion we found that WT and \textit{rms1} buds responded equally well to direct treatment with a high (50 \( \mu \)M) dose of BA (Figure 2B). When applied directly to WT and \textit{rms1} buds, GR24 treated in combination with BA significantly reduced the BA-induced bud growth (Figure 2B; \( p<0.01 \)). Again, as expected, bud growth in SL insensitive \textit{rms4} plants treated with or without BA showed no inhibition by GR24 treatment. It therefore appears that \textit{RMS4}-dependent SL signalling and CK act antagonistically to regulate bud growth.

To determine if the apparent antagonistic relationship between SL and CK in control of bud growth functions in a classical dose-dependent manner expected of hormone signalling, dose-response experiments were performed. We used \textit{rms1} SL deficient plants for these experiments because their buds show a growth response to vascular supply of BA, and to GR24 (e.g. Gomez-Roldan et al., 2008). We again utilised 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 (Figure 3A). Increasing concentrations of GR24 resulted in a dose-dependent reduction of BA-induced bud growth (Figure 3A). Likewise, increasing concentrations of BA supplied to the vasculature decreased the bud inhibiting effectiveness of GR24 (Figure 3B). It therefore appears that GR24 and BA act antagonistically in a dose-dependent manner on bud growth when both supplied to the vasculature.

Since buds of WT plants (which have endogenous SLs) did not respond much to vascular supply of BA in comparison to 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 hypothesised 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 conducted using the *rms1* SL deficient mutant such 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 (Figure 4A; p<0.01) and that GR24 applied to the bud was effective at inhibiting growth induced by vascular supply of BA (Figure 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 (Figure 4A). It is therefore unlikely 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 (Figures 1-4 and 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 expression of *PsBRC1* in buds treated with BA, GR24 or BA and GR24 (Figure 5)
is negatively correlated with bud growth (Figure 2B). As seen in Braun et al. (2011), expression of \( PsBRC1 \) was decreased in SL mutant buds compared to WT buds, addition of BA reduced expression of \( PsBRC1 \) in buds of WT and SL mutant plants, while addition of GR24 increased expression of \( PsBRC1 \) in buds in a \( RMS4 \)-dependent manner (Figure 5). Combined treatment of BA and GR24 resulted in an intermediate level of \( PsBRC1 \) expression in WT and \( rms1 \) buds (Figure 5), which correlates nicely with the intermediate growth responses observed for this treatment combination (Figure 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 regulate of \( PsBRC1 \) expression directly without the need for \textit{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; e.g. Koshiba et al., 1995; Arite et al., 2007). Treatment of WT and \( rms1 \) SL deficient buds with CHX alone caused respective 77% and 69% increases in \( PsBRC1 \) transcript abundance (Figure 6), indicating that transcription of \( PsBRC1 \) is normally repressed by a rapidly turned over protein or that the \( PsBRC1 \) transcript is normally destabilised by a rapidly turned over protein. Reduction of protein synthesis by CHX treatment did not prevent BA from reducing expression of \( PsBRC1 \) (Figure 6A), indicating that 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 expression of \( PsBRC1 \) (Figure 6B). These data suggest that CK and SL may interact quite closely with \( PsBRC1 \) or with stable proteins which in turn interact directly with \( PsBRC1 \) to control bud outgrowth in pea.

Physiological experiments show that although buds of WT 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 (Figures 1A, 2A and S1A). To determine if this small increase in bud length or lack of sustained bud growth in WT is due to \( PsBRC1 \) not being regulated by vascular-derived BA, \( PsBRC1 \) expression was examined in WT buds 24 h after BA treatment either directly to the bud or to the vasculature (Figure 7). Supplying BA to the vasculature or to the buds directly caused comparable reductions in expression of \( PsBRC1 \) in the bud (Figure 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, but as found previously (Figures 1A, 2A and S1A) WT buds did not show continued growth when BA was supplied to the vasculature compared to direct treatment (data not shown). These data suggest that the difference in the longer-term growth response between WT buds treated directly with BA to those treated from a distance is not due to failure to initiate molecular responses in the bud. It also suggests that CK
supplied to the vasculature does indeed make it to the bud and that the small increase in WT bud size after BA is supplied to the vasculature (e.g. Figures 1A, 2A, 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; Beveridge et al., 1997b; Foo et al., 2007; Beveridge et al., 2009; 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 expression of CK biosynthesis genes, *PsIPT1* and *PsIPT2* expression was observed in tissue containing internode and node 3 (including bud) from WT, *rms1* SL deficient and *rms4* SL response mutants (Figure 8). Interestingly, expression of *PsIPT1*, but not *PsIPT2*, was increased in *rms1* and *rms4* SL mutants compared to WT plants, consistent with the possibility that SLs negatively regulate expression of CK biosynthesis genes in the shoot.

**CK biosynthesis genes are not affected by GR24 in WT isolated segments**

To determine if the increase in *PsIPT1* CK biosynthesis gene expression observed in the pea SL mutants (Figure 8) is directly due to loss of SL response, we tested if the synthetic SL GR24 can regulate expression of *PsIPT1* or *PsIPT2* within a short time-frame by incubating isolated segments from internode 4 of young WT pea seedlings with or without 1 µM GR24 and/or 10 µM IAA for 4 hours (Figure 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, expression of *PsIPT1* and *PsIPT2* was increased in isolated segments incubated in control buffer compared to that observed in intact plants, as a segment has no apical source of auxin (Figure 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 expression of *PsIPT1* or *PsIPT2*. We therefore conclude that any regulation of *PsIPT* expression by SL is either indirect (that is, takes longer than 4 h) or is specific to node or bud tissues, which were not tested here.

**DISCUSSION**

**SL mutants show increased response to local and long-distance vascular 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 response of WT and SL mutant plants to CK. SL mutant buds showed increased bud growth responses to CK (6-benzylaminopurine, BA or trans-zeatin riboside, tZR) treatments than WT buds when CK was supplied to the stem (Figure 1A, 2 and S1A). At the node above the site of supply, WT buds showed a poor response to CK (BA and tZR) supplied to the vasculature, increasing up to
only a few millimetres in length. It is unlikely that higher concentrations of CK supplied to the stem would induce increased outgrowth of WT buds, as the highest tested concentrations of CK appeared toxic to the plant, leading to stem thickening and reduced overall plant height (Figure S1B). WT buds did however grow in response to BA supplied locally to the bud (Figure 1B, 2B), but SL mutant buds showed increased bud growth responses at lower concentrations of applied BA (Figure 1B).

The increased branching phenotypes of CK overproducing plants, for example *altered meristem program 1* (*amp1*; Chaudhury et al., 1993), are likely due to increases in CK local to the bud. Other studies have found long-distance supply of CK from rootstocks of CK overproducing 35S-*ipt* transgenic lines are not able to increase branching in non-transgenic WT shoots (Faiss et al., 1997). This is consistent with our finding that WT buds show a poor response to vascular 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 WT 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 WT results in less delivery of CK to the bud compared to that in SL mutants due to some indirect effect such as a difference in water supply to or transpiration of WT and mutant buds; indeed non-growing SL mutant buds were larger than corresponding non-growing WT buds (see size of control treated buds in Figures 1, 2 or 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 (Figure 1A). Alternatively, it could be that SL mutant buds are at a more responsive stage than WT buds due to a more direct consequence of reduced SL signalling (Dun et al., 2006). Grafting experiments in pea suggest that long-distance supply of CK in the xylem is only effective at inducing branch growth of buds that are already released from inhibition by SL (Dun et al., 2009b).

**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 (Figure 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 (Figures 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 (Figures 2B, 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; Beveridge et al., 1997b; Foo et al., 2007; Beveridge et al., 2009; 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-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 WT (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 to WT 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), expression of PsiIPT1, but not PsiIPT2, is elevated in SL mutants (Figure 8). This increased PsiIPT1 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 PsiIPT1 is likely a long-term consequence of SL deficiency/insensitivity rather than a direct effect, as GR24 had no effect of PsiIPT1 expression within 4 h in an isolated stem segment assay (Figure 9). It must be noted that the effect of GR24 on PsiIPT1 expression was only tested in an in vitro system using WT segments, which should be SL deficient prior to GR24 treatment due to removal of the apical supply of auxin (e.g. Foo et al., 2005), and which may behave differently to intact plants (Dun et al., 2006). Nevertheless, even in auxin treated segments, where PsiIPT gene expression was restored to near WT levels, no effect of GR24 on PsiIPT 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 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 be their primary function in bud outgrowth control. Rather, physiological data indicates increased CK sensitivity in SL mutants, and as discussed below, GR24 can decrease, and BA can increase, the expression of PsBRC1 in the bud (Figure 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 regulation of PsBRC1 expression in the bud.
Several observations support the premise that \textit{TB1} and \textit{BRC1} play an important and integral role in bud outgrowth regulation, acting specifically at the axillary bud. Expression levels of \textit{TB1}, a monocot homolog of \textit{BRC1}, were previously demonstrated to underlie differences in branching between maize (\textit{Zea mays} spp. \textit{mays}) and its ancient progenitor teosinte (\textit{Zea mays} spp. \textit{parviglumis}) (Doebley et al., 1997). Additionally, \textit{tb1} and \textit{brc1} mutants in various species have increased branching phenotypes, and \textit{TB1} and \textit{BRC1} are expressed predominantly in axillary buds (e.g. Takeda et al., 2003; Aguilar-Martínez et al., 2007; Finlayson, 2007; Minakuchi et al., 2010; Braun et al., 2011).

The expression of \textit{TB1} and \textit{BRC1} appears to be under close hormonal control. \textit{TB1} and \textit{BRC1} expression is reduced in buds of Arabidopsis and pea SL mutant plants (Figure 5; Aguilar-Martínez et al., 2007; Finlayson, 2007; Braun et al., 2011), and \textit{BRC1} expression in pea is increased by exogenous treatment with the synthetic SL GR24 (Figure 5, Figure 6B; Braun et al., 2011). Additionally, where tested, treatments which promote bud outgrowth such as decapitation, which affects the level of several plant hormones, or CK treatment, decrease \textit{TB1} and \textit{BRC1} expression in rice, pea and Arabidopsis, respectively (Figure 5, Figure 6A, Figure 7; Aguilar-Martínez et al., 2007; Minakuchi et al., 2010; Braun et al., 2011).

We have further dissected the integration of SL and CK signalling by \textit{BRC1} by examining the effects of single and joint applications of these hormones on bud outgrowth and on gene expression and by determining whether each hormone acts directly or indirectly on regulating \textit{PsBRC1} expression. We found that the expression of \textit{PsBRC1} in axillary buds of pea a short period of 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 (Figure 2B, Figure 5). This supports the premise that SL and CK regulate bud outgrowth via regulation of this gene in the bud.

We also found that expression of \textit{PsBRC1} and bud dormancy markers in buds of WT plants treated with BA to the vasculature or bud correlates with the current bud growth status at the time of harvest (Figure 7). Further research is required to determine why buds of WT plants supplied with BA to the vasculature do not continue growth into a branch.

By treatment with the translation inhibitor cycloheximide (CHX; e.g. Koshiha et al., 1995; Arite et al., 2007), we show that GR24 and BA regulation of \textit{PsBRC1} expression does not require protein synthesis (Figure 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 \textit{PsBRC1}.

\textbf{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 \textit{PsBRC1} (Figure 5). This decreased expression of \textit{PsBRC1} relative to WT would essentially prime the bud for an enhanced response to CK whilst 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 in size than the corresponding WT buds (Figure 1, 2 or S1). We therefore expect that there is a certain level or threshold below which \textit{PsBRC1} expression must drop before bud outgrowth ensues. This would in large enable the quantitative integration of multiple factors, hormonal, developmental and environmental, that affect bud outgrowth (Kebrom et al., 2006; Aguilar-Martínez et al., 2007; Finlayson, 2007; Kebrom et al., 2010; Minakuchi et al., 2010; Braun et al., 2011).

Exactly how \textit{BRC1} and \textit{TB1} function as integrators of many signals controlling bud outgrowth is not yet understood. Being members of the TCP protein family, \textit{BRC1} and \textit{TB1} possess a basic helix-loop-helix motif which facilitates DNA binding and likely function as transcriptional activators or repressors (reviewed in Martín-Trillo and Cubas, 2010). Some TCPs in the same class as \textit{BRC1} and \textit{TB1} are involved in cell cycle progression (Masuda et al., 2008), while others might regulate transcription of cell cycle marker genes (e.g. Nath et al., 2003; reviewed in Martín-Trillo and Cubas, 2010). In contrast, a TCP family member has also been potentially implicated in a non-transcriptional control process involving protein-protein interactions (Suzuki et al., 2001). Further studies are required to elucidate how modulation of \textit{BRC1} and \textit{TB1} expression translates to 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 for theories on shoot branching**

The antagonism between SL and CK in bud outgrowth control might explain conflicting findings with regards to SL-inhibition of bud outgrowth after decapitation or segment isolation and which 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 (reviewed in Domagalska and Leyser, 2011). Alternatively, although not necessarily exclusively, Brewer et al. (2009) suggest that SLs may act directly in axillary buds. This later hypothesis is supported here by characterisation of a bud specific gene, \textit{BRC1}, which is essential for bud growth suppression and which is tightly regulated at the level of gene expression by SL and CK. In addition to the bud specific expression of \textit{BRC1}, the SL and CK interaction in buds is supported by physiological evidence that vascular supplied hormones interact equally well with locally supplied or vascular supplied hormones to antagonistically influence bud outgrowth (Figures 3 and 4) and that both local and vascular supplied CK can affect \textit{PsBRC1} expression (Figure 7).
Using this second hypothesis, we can re-interpret findings on SL responses in isolated stem segments and decapitated plants. In both circumstances, auxin levels are depleted in the adjacent stem to the axillary bud and 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 WT, 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 repression of bud growth by basal supply of SL in the media (Crawford et al., 2010; Liang et al., 2010) supporting the auxin transport model. By contrast, SL treatment direct 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 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 CK content in these different systems to gauge the impact for 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 localisation 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 SL function is now emerging to be important in other developmental processes, such as root development, it is possible that its effect on auxin transport may be more important for those processes rather than for shoot branching.

CONCLUSION

We show that expression of PsBRC1 is tightly regulated by SL and CK, and that the level of expression correlates with bud outgrowth responses. Results presented here support a model in which SL up-regulates expression of PsBRC1 while local CK represses its expression; 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, *Pisum sativum* (pea) plants were grown two per two litre 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, Australia) were supplied weekly. For gene expression studies in Figures 5, 6, 7 and 9, plants were grown at six, six, three or three, respectively, per two litre 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 WT cultivar 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 callipers. Plant height was measured from node 1 to the shoot apex.

For statistical analyses, 1-way ANOVA with Tukey post-test and Students t-tests were performed using GraphPad Prism v 5.01 (GraphPad Software, Inc.; www.graphpad.com).

**Hormone and chemical treatments**

6-Benzylaminopurine (BA), *trans*-zeatin riboside (tZR) and the synthetic SL GR24 were supplied to the main stem vasculature below node 3, or applied directly to the bud at node 3, as described in Gomez-Roldan et al. (2008) and Dun et al. (2009b), respectively, except solutions contained 0.09% dimethyl sulfoxide (DMSO) for the experiment including tZR treatments (Figure S1). For Figures 5, 6 and 7, treatments, including those with cycloheximide (CHX), were applied to the bud at node 3 as described above, but solutions also contained 0.1% DMSO.

**In vitro Hormone Treatments**

Individual biological replicates consisting of seven 12 mm segments of stem tissue from internode 4 were incubated in 9 mL 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 in Dun et al. (2009b) (Figures 8 and 9), and bud tissue as described in Braun et al. (2011) (Figures 5, 6 and 7). cDNA synthesis and real-time PCR gene expression analyses were performed as described in Dun et al. (2009b). Primer sequences were as follows: *PsIPT1* forward (5’-ACCGTCTTGTGCTACGAGGTTGTGC-3’) and *PsIPT1* reverse (5’-TCTAATGGTTACCCCTGACAGACG-3’) (Tanaka et al., 2006); *PsIPT2*
forward (5’-TGGCAGCAACATCATCCTCTG-3’) and PsIPT2 reverse (5’-ACCTGTGGCCCCATTACCAT-3’) (Tanaka et al., 2006); PsBRC1 forward (5’-TCGAAAGACGGAATCAACATAA-3’) and PsBRC1 reverse (5’-TCCCTTGCTTTTCTCTTTG-3’); PsDRM1 forward (5’-CCCTCAAGATGGAGGAGCGG-3’) and PsDRM1 reverse (5’-CAACTTTACGCGCCATGAGG-3’); PsBRC1 forward (5’-TGTCATTCTTGGCCTATTG-3’) and PsBRC1 reverse (5’-TCCCTTGCTCTTCTTGTG-3’); PsDRM1 forward (5’-CCCTCAAAGATGATGGAGCG-3’) and PsDRM1 reverse (5’-CCACCCAGTCCATAA-3’); PsAD1 forward (5’-GGTGGTGTTGTTGTTTCAAT-3’) and PsAD1 reverse (5’-TCCGCAGCAGTGAAGACT-3’); 18S forward (5’-ACGTCCGTGCCTTTGTACG-3’) and 18S reverse (5’-CACTTCACGGACCATTCAAT-3’) (Ozga et al., 2003); EF1α forward (5’-TGTGCCAGTGGGACGTGTTG-3’) and EF1α reverse (5’-CTCGTGGTGCGATCTCAACGG-3’). In all cases, error bars represent biological standard error.

SUPPLEMENTAL MATERIAL

Figure S1. Buds of WT plants are less responsive to vascular supply of trans-zeatin riboside (tZR), a CK found endogenously in xylem sap, and 6-benzylaminopurine (BA) than SL deficient plants (A), and increasing concentrations of tZR and BA reduces WT and SL mutant plant height (B).

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FIGURE LEGENDS

Figure 1. SL deficient plants are more responsive to CK than WT plants. The synthetic CK 6-benzylaminopurine (BA) was supplied to the stem vascular stream below node 3 (A) or applied directly to the bud at node 3 (B) of 9-day-old WT and rms1-2 (Torsdag) pea plants. Bud length at node 3 was measured 7 days after treatment. Data are means ± standard error (n=7-12).

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) was (A) supplied to the vasculature below node 3 or (B) applied directly to the bud at node 3 of 10-day-old WT, rms1-2 or rms4-1 (Torsdag) pea plants. Bud growth at node 3 was measured 7 days after treatment. Data are means ± standard error (n=7-12).

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 (A) 9-day old and (B) 10-day-old rms1-2 (Torsdag) pea plants. Bud length at node 3 was measured 7 days after treatment. Data are means ± standard error (n=14 (A) and 9-12 (B)).

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

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

Figure 6. SL and CK regulate PsBRC1 without the need for de novo protein synthesis. The bud at node 3 of 8-day old WT (A) and rms1-2 (B) (Torsdag) pea plants was treated for 6 h with or without the synthetic SL, 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 ± standard error (n=3 pools of 30 plants), and are from the same experiment as Figure 5.
Figure 7. CK supplied locally or from a distance decreases the expression of *PsBRC1* and bud dormancy markers 24 h after treatment. 0 or 50 µM BA was supplied to the vasculature below node 3 or applied directly to the bud at node 3 of 9-day-old WT 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 ± standard error (*n*=3 pools of 28-30 plants).

Figure 8. *PsIPT1* but not *PsIPT2* expression is increased in SL mutant stem tissue. Internode 3 and node 3 (including bud) was harvested from 12-day old (4-5 leaves expanded) WT, *rms4-1* and *rms5-3* (Torsdag) pea plants. Expression is represented relative to WT; *18S* was used as the internal reference gene. Data are means ± standard error (*n*=7-8).

Figure 9. The synthetic SL, GR24, does not affect *PsIPT1* or *PsIPT2* expression in isolated WT stem tissue segments within 4 h. 12 mm segments from internode 4 of 12-day old WT Torsdag pea plants were incubated for 4 h with 0 or 1 µM GR24, 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 ± standard error (*n*=3 pools of 7 plants).

Figure S1. Buds of WT plants are less responsive to vascular supply of *trans*-zeatin riboside (tZR), a CK found endogenously in xylem sap, and 6-benzylaminopurine (BA) than SL deficient plants (A), and increasing concentrations of tZR and BA reduces WT and SL mutant plant height (B). tZR or BA was supplied to the vascular stream below node 3 of 10-day-old WT and *rms1-2* (Torsdag) pea plants. Bud growth at node 3 (A) and plant height (B) was measured 7 days after treatment. Data are means ± standard error (*n*=11-12).
Figure 1. SL deficient plants are more responsive to CK than WT plants. The synthetic cytokinin 6-benzylaminopurine (BA) was supplied to the stem vascular stream below node 3 (A) or applied directly to the bud at node 3 (B) of 9-day-old WT and rms1-2 (Torsdag) pea plants. Bud length at node 3 was measured 7 days after treatment. Data are means ± standard error (n=7-12).
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