PsBRC1 and shoot branching in pea

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DEVELOPMENT AND HORMONE ACTION
The pea TCP transcription factor PsBRC1 acts downstream of strigolactones to control shoot branching.1

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Abstract:

The function of PsBRC1, the pea (Pisum sativum L.) homolog of the maize TEOSINTE BRANCHED1 (TB1) and the Arabidopsis BRANCHED1 (AtBRC1) transcription factors was investigated. The pea Psbrc1 mutant displays an increased shoot branching phenotype, is able to synthesize strigolactone (SL) and does not respond to SL application. The level of pleiotropy of the SL deficient rms1 mutant is higher than in the Psbrc1 mutant, rms1 exhibiting a relatively dwarf phenotype and more extensive branching at upper nodes. The PsBRC1 gene is mostly expressed in the axillary bud and is transcriptionally upregulated by direct application of the synthetic SL GR24 and downregulated by the cytokinin (CK) 6-benzylaminopurine (BAP). The results suggest that PsBRC1 may have a role in integrating SL and CK signals and that SLs act directly within the bud to regulate its outgrowth. However the Psbrc1 mutant responds to BAP application and decapitation by increasing axillary bud length implicating a PsBRC1-independent component of the CK response in sustained bud growth. In contrast to other SL-related mutants, the Psbrc1 mutation does not cause a decrease in the CK zeatin riboside (ZR) in the xylem sap nor a strong increase in RMS1 transcript levels suggesting that the RMS2-dependent feedback is not activated in this mutant. Surprisingly the double rms1 Psbrc1 mutant displays a strong increase of numbers of branches at cotyledonary nodes whereas branching at upper nodes is not significantly higher than the branching in rms1. This phenotype implicates a localized regulation of branching at these nodes specific to pea.
Introduction:

Early studies on shoot branching were based on decapitation experiments which emphasized the role of the shoot apex in the inhibition of axillary bud outgrowth (Thimann and Skoog, 1933). In the classical theory of apical dominance, auxin from the apex was proposed to act indirectly to suppress bud outgrowth while cytokinin (CK) coming from the roots promoted bud outgrowth (Snow, 1937; Sachs and Thimann, 1967; Cline, 1991). More than a decade ago, with the identification and characterization of high branching mutants in pea, Arabidopsis, rice and petunia, long-distance signaling was shown to be an important process in the control of shoot branching (Ongaro and Leyser, 2008; Beveridge et al., 2009; McSteen, 2009; Beveridge and Kyozuka, 2010). In pea, grafting revealed the existence of two novel long distance signals controlling shoot branching that were different from auxin and cytokinin (Beveridge et al., 2000; Beveridge, 2006; Beveridge et al., 2009): a root-to-shoot branching inhibitor (Beveridge et al., 1997a; Morris et al., 2001) which was subsequently identified as a strigolactone (SL) or derived compound (Gomez-Roldan et al., 2008; Umehara et al., 2008) and a shoot-to-root feedback signal, which was shown to be auxin independent and has still to be identified (Beveridge et al., 1997b; Beveridge et al., 2000). To date the genetic and physiological model of branching control in pea includes five RAMOSUS genes (RMS1 to RMS5). Branching of the pea SL deficient rms1 and rms5 mutants is suppressed when scions of these mutants are grafted on WT rootstock or when the synthetic SL GR24 is applied on axillary buds. In contrast, the rms3 and rms4 SL response mutants are not rescued when grafted to WT rootstocks, and they do not respond to GR24 application (Table 1) (Beveridge et al., 1996; Beveridge et al., 2009; Dun et al., 2009). All rms mutants, except rms2, have high levels of RMS1 transcripts in epicotyls compared to WT (Foo et al., 2005). Moreover these branching mutants, with the exception of rms2, have greatly reduced amounts of CK in xylem sap (X-CK) compared with WT plants (Table 1) (Beveridge et al., 1997b; Morris et al., 2001; Foo et al., 2007). The reduced X-CK in several rms branching mutants appears to be mediated by a shoot-to-root mobile signal (Beveridge et al., 1997b; Beveridge, 2000). Because rms2 is the only rms mutant that does not show down-regulation of X-CK, it has been proposed that RMS2 may play a role in the generation of this feedback signal. It was hypothesized that the same signal may also regulate RMS1 transcript levels (Foo et al., 2005) because X-CK and RMS1 transcript levels are typically anticorrelated (Dun et al., 2009). The
*rms2* mutant does respond to SL (Dun et al., 2009). This mutant having low transcript levels of *RMS1* and slightly elevated X-CK in comparison with WT may branch because of low SL levels and/or high CK content.

Among these five *RMS* genes, three have been cloned and correspond to branching genes identified in other species. The *RMS1* and *RMS5* genes encode the CAROTENOID CLEAVAGE DIOXYGENASE (CCD) enzymes, CCD8 and CCD7 respectively (Sorefan et al., 2003; Johnson et al., 2006). The *RMS4* gene encodes an F-Box protein and corresponds to the Arabidopsis *MAX2* gene (Stirnberg et al., 2002; Johnson et al., 2006). While *RMS4* transcripts are found in all tissues, the SL biosynthesis *RMS1* and *RMS5* genes are highly expressed in roots and in the basal stem (Foo et al., 2005; Johnson et al., 2006) and were shown to be finely regulated along the stem (Dun et al., 2009).

In the current model of shoot branching in pea, auxin, originating from the main shoot apex regulates SL levels by maintaining *RMS1* and *RMS5* transcript levels (Foo et al., 2005; Johnson et al., 2006) and down-regulates CK in both xylem sap (Bangerth, 1994; Li et al., 1995) and in the stem (Tanaka et al., 2006). Auxin regulation of SL synthesis genes, and bud outgrowth inhibition by SL applications in decapitated plants suggest that SL is the second messenger by which auxin controls branching in decapitation experiments (Brewer et al., 2009). SL and CK would act locally within the axillary bud to control its outgrowth. Another hypothesis has been proposed where SLs would act upstream of auxin by modulating its transport in the main shoot by limiting accumulation of PIN auxin efflux carrier protein on the plasma membrane of cells involved in the polar auxin transport stream (PATS) (Domagalska and Leyser, 2011). Consistent with this, the Arabidopsis branching *max* mutants show increased polar PIN accumulation and increased auxin transport (Bennett et al., 2006; Prusinkiewicz et al., 2009; Crawford et al., 2010). The rice SL deficient *d27* mutant also shows increased auxin transport (Lin et al., 2009) but this difference is not always observed in pea (Beveridge, 2000; Beveridge et al., 2000; Beveridge, 2006; Dun et al., 2006). In the auxin transport model, the auxin exported from active apices, moving in the PATS, would prevent auxin export from dormant buds, and therefore would block their outgrowth, this process being amplified by the canalisation positive feedback. Buds would compete to export auxin into the main stem, and SL by dampening auxin transport in the PATS would enhance this competition (Crawford et al., 2010; Domagalska and Leyser).

How SL and CK interact to control axillary bud outgrowth is still not understood and the role of auxin is still a matter of debate. However, the discovery of SL as a plant hormone allows novel approaches. In particular, discovery of genes responding to SL application and
deciphering SL signaling pathways are essential for a better understanding of the control of branching. Axillary buds have to integrate many factors that influence switching between dormant and growing states during plant ontogeny and they can respond differently according to their position along the main stem (Cline, 1991). It is very likely that several pathways control axillary bud outgrowth and that important molecular processes within the bud are involved in this control in particular to integrate the multiple long distance signals. In this paper, PsBRC1, the pea homolog of TEOSINTE BRANCHED1 (TB1) from maize (Doebley et al., 1997) and of BRANCHED1 (AtBRC1) from Arabidopsis (Aguilar-Martinez et al., 2007; Finlayson, 2007) was integrated into the pea model. This gene is almost exclusively expressed in the axillary bud and may provide the link between systemic signaling and events occurring within the axillary bud to control bud outgrowth.

The TB1 gene from maize, which affects plant and inflorescence architecture, is a well-known target for artificial selection during maize domestication from its wild and highly branched ancestor teosinte. It is a striking example of how human selection modified gene expression to change plant architecture. In maize, repression of branching results from higher TB1 transcript levels in axillary buds of maize in comparison with those in teosinte (Doebley et al., 1997; Wang et al., 1999). Recently it has been demonstrated that the corresponding gene in barley (INTERMEDIATE-C) has also been targeted in human selection to regulate not only tillering but also fertility of the lateral spikelet (Ramsay et al., 2011). In monocots, only one homolog of the TB1/CYC clade has been identified that regulates axillary bud outgrowth, whereas in Arabidopsis, two homologs (AtBRC1 and AtBRC2) were shown to control shoot branching. These genes belong to the TCP family of transcription factors specific to plants and named from the first three identified members, TB1, CYCLOIDEA (CYC) in Antirrhinum majus and PCF-coding genes in rice. Phylogenetic analyse of this family comprising 24 members in Arabidopsis (Martin-Trillo and Cubas, 2010) has identified two distinct classes, classes I and II, with roles in plant development and morphogenesis processes as diverse as establishment of floral symmetry, plant architecture (Doebley et al., 1997; Aguilar-Martinez et al., 2007), leaf morphogenesis (Nath et al., 2003; Palatnik et al., 2003; Ori et al., 2007) and senescence (Schommer et al., 2008), embryo growth (Tatematsu et al., 2008), and circadian rhythm (Pruneda-Paz et al., 2009; Giraud et al., 2010). Globally class I TCP proteins appear to promote cell division whereas class II proteins, containing TB1/AtBRC1, repress organ growth by inhibiting cell proliferation (Martin-Trillo and Cubas, 2010). For example class I TCP proteins, PCF1 and PCF2, were shown to promote PCNA (Proliferating Cell Nuclear Antigen) gene expression, activating G1 to S transition by binding
to cis-acting elements in the promoter of PCNA (Kosugi and Ohashi, 1997). Class II TCP proteins, CINCINNATA (CIN) in Antirrhinum and TCP4 in Arabidopsis control leaf morphology by repressing cell proliferation specifically in the leaf margins (Nath et al., 2003; Palatnik et al., 2003; Crawford et al., 2004). In yeast TCP4 has been shown to function in a dose dependent manner and to block cell cycle at G1 to S transition (Aggarwal et al., 2011).

In rice, the high tillering fine culm1 (fc1) mutant, is mutated in the rice homolog of TB1 (Takeda et al., 2003; Arite et al., 2007). Both the absence of response of the fc1 mutant to GR24 application, and the similarity of the phenotype of fc1 d17 double mutant to the SL deficient d17 mutant phenotype, suggested that FC1/OsTB1 acts downstream of the SL pathway in rice (Minakuchi et al., 2010). Here we show that the regulation of pea PsBRC1 exhibits several similarities and differences to those described in rice and Arabidopsis. PsBRC1 is strongly transcriptionally upregulated by SL, which is not the case for the rice FC1 (Minakuchi et al., 2010). In contrast to Arabidopsis (Aguilar-Martinez et al., 2007), branching is increased in the pea Psbrc1 mutant in response to decapitation and to CK application. We propose that PsBRC1, acting in axillary buds, may integrate at the transcriptional level the SL and the CK pathways to regulate axillary bud outgrowth. However the response to decapitation and to direct CK application of the Psbrc1 mutant also suggests a PsBRC1-independent component of the CK response in pea.

Results

1- The pea Psbrc1 mutant phenotype shows that PsBRC1 inhibits bud outgrowth

To isolate a pea homolog of TB1/AtBRC1, the sequence of the Lotus japonicus CYCLOIDEA 5 gene, LjCYC5, (GenBank accession Number DQ202478; (Feng et al., 2006)) was used as it was the closest homolog to Arabidopsis TCP18/AtBRC1. A 400 bp pea homolog sequence was first amplified with degenerate primers. The complete sequence was obtained using PCR walking and RACE PCR (GenBank accession Number JF274232, BankIt1431647) (see Materials and Methods).

Phylogenetic analysis based on the protein sequences of the TCP domain of several members of both the CYC-TB1 group and the PCF group placed the pea sequence in the same clade as TB1 and AtBRC1 and consequently, the gene was named PsBRC1 (see Supplemental Fig. S1). The single intron in PsBRC1 deduced from alignment of the genomic and the cDNA sequences was located at the place of the last intron in AtBRC1 (data not shown). PsBRC1
was then mapped using the RIL population (Térèse x Torsdag) (Laucou et al., 1998) to the pea linkage group IV close to Fa in a region where no RMS genes were ever mapped (Rameau et al., 1998). Consequently, a TILLING approach was initiated using the mutagenized population from the genotype Caméor (Triques et al., 2007; Dalmais et al., 2008). The genomic sequence comprising the two conserved TCP and R domains was screened for mutations (Fig. 1A). Ten mutations in PsBRC1 were identified, 3 giving a silent mutation, one in the intron and 6 leading to a change of amino acid (see Table S1). Among these 6 mutations, only one gave a clear branching phenotype co-segregating with the mutation (family 4654). In all segregating populations this mutation always co-segregated with a strong branching phenotype as in the BC2-F2 (4654 x Caméor) where the 14 Psbrcl mutant plants displayed a thin stem with a strong branching phenotype at nodes 1 and 2 in contrast to the 9 WT plants which had a small branch only at node 2 (see Fig. S2A). This mutation was located in the TCP domain and resulted from a threonine to isoleucine amino acid change in the Loop/helix II transition (T195I). These data strongly support the hypothesis that the T195I mutation in the PsBRC1 gene was the cause of the high branching phenotype.

The mutant plant from the family 4654 was backcrossed 3 times with the Caméor parent line (BC3 (4654 x Caméor) denoted PsbrclCam) and twice with the WT Térèse line in which all other branching mutations are available (mutant line denoted PsbrclTe; see Materials and Methods). The phenotype of PsbrclCam was first compared to its WT progenitor Caméor. Strong basal branching at nodes 1 and 2 was observed in the PsbrclCam mutant with often two branches at node 2 whereas the WT Caméor sometimes showed only a single branch at node 2. Branching at upper nodes (above node 3) was very low in PsbrclCam (Fig. 1B- Fig. 1C upper diagram). The low branching at upper nodes observed in PsbrclCam was particularly evident in the PsbrclTe mutant and was one major difference from the rmsl mutant which showed, in comparison, long branches at each node (Fig. 1C lower diagram and see Supplemental data Fig. S2D). Another difference between the mutants was that plant height was only slightly reduced for PsbrclCam and PsbrclTe in comparison with their respective WT whereas rmsl showed a strong reduction in internode length (Fig. 1B; see Supplemental Fig. S2B). The width of the main stem was reduced in both rmsl and PsbrclCam mutants (see Supplemental Fig. S2C).

rmsl Psbrcl double mutant plants were obtained from a cross between the rmsl mutant derived from the WT Térèse (line M3T-884) and a Psbrcl F2 plant derived from the cross (Térèse x PsbrclCam), also containing the afila mutation (absence of leaflets as in the WT Térèse). In the F2 generation, 103 plants were genotyped for both genes and phenotyped.
A striking feature of double mutant plants was the high number of cotyledonary branches per plant. In this particular genetic background, approximately half of the *Psbrc1* plant displayed cotyledonary branches which is rarely seen for *Psbrc1Cam* in the Caméor genetic background whereas only 4 out of 20 *rms1* plants (that were all heterozygous for *PsBRC1*) branched at this cotyledonary node (Fig. 1D). A more precise phenotyping was performed on 5 or 6 F3 families fixed for *rms1*, *Psbrc1* or both mutations in a comparable genetic background (see Material and Methods). As observed previously, at upper nodes, *rms1* plants were significantly more branched than *Psbrc1Te* plants whereas the double mutant genotype was not significantly more branched than *rms1* at these nodes (Fig. S2D; P<0.05 by least significant difference (LSD) test). At node 2, total branch length was similar in the 3 genotypes whereas at nodes below, and particularly at cotyledonary node, branching of *rms1* *Psbrc1* double plants was significantly higher than in either single mutant (Fig. S2D). Consequently it appeared that at basal nodes, a transgressive phenotype was observed in the double mutant whereas at upper nodes, branching of the double mutant was not significantly different from that observed for *rms1*.

2- *PsBRC1* is a target in the SL signaling pathway.

To test whether *PsBRC1* was expressed mainly in axillary buds, as shown in maize, Arabidopsis, rice and tomato (Hubbard et al., 2002; Takeda et al., 2003; Aguilar-Martinez et al., 2007; Martin-Trillo et al., 2011), WT plants were dissected into different organs/parts and *PsBRC1* transcript levels were quantified by real-time PCR. Transcripts were only detected in axillary buds, floral buds, nodal tissue and the shoot apex. Maximum transcript levels were found in axillary buds. Floral buds, shoot apex and nodal tissue contained very low levels of transcripts, 100 to 1000 times less than in axillary buds (see Supplemental Table S2).

The response of the *Psbrc1Cam* mutant to SL was analysed by application of the synthetic strigolactone GR24 (500 nM) to the axillary bud at node 3 of both *Psbrc1Cam* and *rms1* mutants and their respective WT. Bud length was measured 10 days later (Fig. 2A). No inhibitory effect of GR24 was observed for the WT Térèse and Caméor in which axillary buds are very small at this node. In contrast to the strong inhibition of axillary bud outgrowth in the SL deficient *rms1* mutant, no significant effect of GR24 was observed for the *Psbrc1Cam* mutant (Student’s t-test, P = 0.89). Grafting experiments confirmed the SL insensitivity of *Psbrc1Cam* as branching was not inhibited when *Psbrc1Cam* mutant scion was grafted on WT rootstock in contrast to the SL deficient *rms1* scion grafted on WT rootstock (see Supplemental Fig. S3). *Psbrc1Cam* rootstocks were able to inhibit branching in *rms1* scion
indicating that Psbrc1Cam very likely produces SL and/or the active derived compound (see Supplemental Fig. S3). To confirm that Psbrc1Cam was able to synthesize SL, we quantified SL in root exudates of Psbrc1Cam, Psbrc1Te and their corresponding WT (Fig. 2B). The principal strigolactone detected in root exudates of all genotypes was fabacyl acetate. Amounts of this compound were 3- to 6-fold higher (P<0.05 by Student’s t-test) from both Psbrc1 genotypes than in their corresponding WT background lines (Fig. 2B). Other compounds particularly epi-orobanchyl acetate were detected in most samples but levels were too low for accurate quantitation.

The PsBRC1 transcript level was quantified in axillary buds of the different strigolactone related mutants using real-time PCR (Fig. 3A). Dissected axillary buds were sampled from node 4 of the rms1, rms2 and rms4 mutants and the WT line (Térèse) at a stage when buds had a comparable size (before they start to grow in mutants). PsBRC1 levels were 10 times lower in rms1 and rms4 than in WT (Fig. 3A; see Supplemental Fig. S4A-B). These results indicated that PsBRC1 may act downstream of the SL signaling pathway and downstream of RMS4. In contrast, PsBRC1 transcript level was similar to WT level in axillary buds of the rms2 mutant (Fig. 3A). The rms2 mutant displays strong basal branching in comparison to rms1 (Dun et al., 2009), and at node 4, from where the buds were sampled, the axillary buds of rms2 plants were only 2.3 mm long whereas they reached 14 mm in rms1 (data not shown). The rms2 phenotype of inhibited buds at node 4 is explained by a (basal) branch-derived feedback signal, possibly auxin, which increases RMS1 expression and presumably SL synthesis in rms2 plants (Dun et al., 2009). This SL moves up the stem and because the rms2 mutant is able to synthesize and to respond to SL, it could explain why in this branching mutant PsBRC1 transcript levels were not low at this upper node.

To further characterise the relationship between SL and PsBRC1 expression, PsBRC1 transcript levels were followed from 6 h up to 24 h after SL application (Fig. 3A). Transcript levels increase in axillary buds of WT, rms1 and rms2 after GR24 application (500 nM) but not in rms4 (Fig. 3A and S4A). In WT and rms2 axillary buds a two-fold increase was observed 24 h after GR24 application. In rms1 mutant buds, PsBRC1 mRNA level increased 10 fold 6 h and 24 h after GR24 treatment (Fig. 3A and S4A). In rms4, there was no effect of GR24 treatment with PsBRC1 transcript levels remaining very low throughout (Fig. 3A and S4A). To test if induction of PsBRC1 by GR24 is affected in Psbrc1, the Psbrc1Te mutant was included in one experiment with the WT Térèse, rms1 and rms4. PsBRC1 transcript levels were followed from 6 to 48 hours after GR24 application (Fig. 3B). PsBRC1 transcript levels were very low in rms1 and rms4 axillary buds compared with WT and Psbrc1Te axillary buds.
In several experiments transcript levels of \( PsBRC1 \) were not reduced in the \( Psbrc1^{Cam} \) mutant in comparison to levels in the corresponding WT Caméor (Fig 3B, Fig 4A and Fig S4B), whereas they are reduced in SL deficient and SL response mutants. Again \( PsBRC1 \) transcript levels remained very low in \( rms4 \) whereas a strong increase was observed after GR24 treatment in \( rms1 \). In the WT and \( Psbrc1^{Te} \) mutant, a two-fold increase of \( PsBRC1 \) by GR24 was generally observed in contrast to the non-induction in a \( rms4 \) background (Fig. 3B). All these results indicate that \( PsBRC1 \) transcription and its induction by GR24 are not affected in the \( Psbrc1 \) mutant.

3- CK regulates \( PsBRC1 \) at the transcriptional level independently from SL

To investigate whether CK regulates the transcription of \( PsBRC1 \), axillary buds at node 4 of \( Psbrc1^{Cam} \), \( rms1 \) and \( rms4 \) mutants and their corresponding WT were harvested 6 h after direct application of the synthetic cytokinin 6-benzylaminopurine (BAP) (50 µM) and \( PsBRC1 \) transcript levels were quantified using real-time PCR (Fig. 4A and Fig. S4B). In all genotypes, BAP application led to a strong reduction of \( PsBRC1 \) transcript levels. This included the \( rms1 \) SL deficient and the \( rms4 \) SL response mutants in which \( PsBRC1 \) transcript levels were already low. The CK effect on \( PsBRC1 \) expression in \( rms4 \) buds shows that CK can transcriptionally regulate \( PsBRC1 \) independently of SL.

4- The \( Psbrc1^{Cam} \) mutant responds to exogenous CK application and to decapitation

To test whether \( PsBRC1 \) is needed for axillary bud growth response to cytokinin, CK was applied to bud 4 of WT Térèse and Caméor, \( rms4 \) and \( Psbrc1^{Cam} \) mutants and buds were measured 5 and 7 days after application (Fig. 4B). For all lines, axillary buds did not grow much without CK treatment as in this experiment, to have a better comparison between WT and mutants, the primary bud at upper nodes in mutants was removed prior to the treatment as were the basal lateral branches for all genotypes. All genotypes responded to BAP application by displaying a strong increase in bud/branch length compared with the mock treated buds particularly for \( rms4 \) and \( Psbrc1^{Cam} \) mutants. Another way to test the response to CK is decapitation which by depleting the source of auxin, has been shown to induce a rapid and massive decrease of the \( RMS1 \) SL biosynthesis gene expression, together with rapid increases in CK level in xylem sap and in transcript levels of CK biosynthesis genes in nodal tissue (Bangerth, 1994; Foo et al., 2005; Tanaka et al., 2006; Foo et al., 2007). Decapitation of \( rms1 \)
and Psbrc1Cam mutants and their corresponding WTs resulted in increased branch lengths in all genotypes (Fig. 4C). Because the Psbrc1Cam mutant has been shown to be insensitive to GR24 application, its response to decapitation suggests that this response may be largely due to CK level variation occurring after decapitation or to CK-independent effects of auxin depletion.

5- PsBRC1 and the RMS2-dependent feedback signal

The SL biosynthesis gene RMS1/CCD8 is highly regulated at the transcriptional level. In particular, absence of SL response because of SL deficiency (in rms1, rms5) or lack of response (in rms3, rms4) induces a feedback signal that strongly upregulates RMS1 transcript levels (Beveridge et al., 1997a; Beveridge et al., 1997b; Beveridge, 2000; Foo et al., 2005). The RMS2 gene may control this feedback signal, as RMS1 transcript levels are not upregulated in the rms2 mutant. RMS1 transcript accumulation was quantified in epicotyls from rms1, rms2, rms4 and Psbrc1Cam mutants and their corresponding WT (Fig. 5A and Fig. S4E).

Consistent with previous findings, RMS1 mRNA was more abundant in rms1 and especially in rms4 mutants compared with WT Térèse. In contrast, rms2 and Psbrc1Cam mutants contained 3 and 6 times lower transcript levels compared to their respective WT lines suggesting that the feedback signal is not activated in a Psbrc1 background. As the same feedback signal appears to control CK concentration exported from roots in the xylem-sap (X-CK) (Beveridge et al., 1997b; Beveridge, 2000; Foo et al., 2005; Foo et al., 2007), CK content of xylem sap of WT and Psbrc1Cam plants was measured in two separate experiments (Fig. 5B). The main compounds detected were trans-zeatin riboside (tZR) and dihydrozeatin riboside (DZR) Smaller amount of isopentenyl adenosine (IPR), trans-zeatin (tZ), cis-zeatin riboside (cZR), isopentenyl adenine (IP) and dihydrozeatin (DZ) were also present. The profiles of CKs and absolute amounts were very similar in both experiments, with no statistically significant difference between WT and Psbrc1Cam genotypes (Student’s t-test, P = 0.49). This contrasts with Arabidopsis max and pea rms branching mutants which, with the exception of rms2, have highly depleted X-CK levels (Table 1) (Beveridge et al., 1997b; Foo et al., 2007). Levels of X-CK were also quantified in rms1 Psbrc1 double mutant plants in comparison to WT and single mutant plants derived from the same cross to have a comparable genetic background and to have enough plants for sap collection (see Materials and Methods).
X-CK levels in the F3 rms1 Psbrc1 double mutant plants were not significantly different from X-CK levels from F3 rms1 plants whereas X-CK levels from F3 Psbrc1 and Psbrc1Te line were particularly high in comparison to WT Térèse and F3 WT plants. The greatly increased X-CK in Psbrc1Te in this experiment was not seen in the previous analysis with Psbrc1Cam and its WT Caméor and may relate to genetic background. Overall, these results indicate that the feedback regulation of X-CK is restored in the Psbrc1 rms1 double mutant but is absent or mis-regulated in Psbrc1 single mutants.

Discussion

The phenotype of Psbrc1 differs from the phenotype of the SL deficient rms1 mutant

Despite its large genome and recalcitrance to transformation, pea is an excellent model plant for genetic and physiological studies. The TILLING approach is particularly adapted for reverse genetics in such model plants (Triques et al., 2007; Dalmais et al., 2008) and has been applied in the present work to identify a novel branching mutant in the TCP transcription factor PsBRC1. The Psbrc1 mutant showed a strong branching phenotype in comparison to its WT progenitor Caméor, particularly at basal nodes; buds at upper nodes were larger or gave small branches in comparison with the WT. When compared to the SL deficient rms1 mutant, the Psbrc1 mutant displayed very few long branches at upper nodes. Moreover its height was less affected compared with rms1 and other rms mutants which are relatively dwarf. In rice, the fc1 mutant is also less affected in height and in tiller number than the dwarf (d) SL mutants (Arite et al., 2007). In tomato, the phenotype of SIBRClb RNAi lines are also less strong than the phenotype of the Slccd7 lines (Martin-Trillo et al., 2011) which supports the idea that the milder phenotype of Psbrc1 compared with rms1 is likely not due to a leaky mutation. In addition, the threonine mutated to isoleucine in the studied Psbrc1 pea mutant (T195I) is located in the loop/helix II transition and appears highly conserved in the class I TCP family and in the class II CYC/TB1 clade (Martin-Trillo and Cubas, 2010). The relatively mild branching phenotype could also explain why the intensive screenings for high branching mutants in pea EMS mutagenized populations previously failed to identify the Psbrc1 pea branching mutant.

The difference of phenotypes between the SL deficient rms1 and Psbrc1 mutants may be explained by the very localised expression of the PsBRC1 gene, mostly expressed in
axillary buds in contrast to the SL biosynthesis RMS1 and RMS5 genes that are highly expressed in roots and also significantly in stems where they are regulated by different long distance signals. Novel roles for SL in plant architecture, other than shoot branching, have been recently suggested for dwarfism (Lin et al., 2009) and root architecture (Koltai et al., 2009; Kapulnik et al., 2010; Ruyter-Spira et al., 2010). The relative dwarfism of rms mutants (in rice the SL mutants were originally called dwarf) is not yet understood but it is very likely that SL controls stem growth, and this control may be independent from PsBRC1. In the auxin transport theory it is proposed that SL modulates polar auxin transport (PAT) in the stem and that axillary buds compete for exporting auxin into the PAT stream in the main stem. The Psbrc1 mutant appears less branched at upper nodes than the SL deficient rms1 mutant which could suggest that competition between buds is higher in the Psbrc1 mutant compared to the other rms mutants. In pea, differences in auxin transport between SL-related mutants and WT are modest (Beveridge et al. 2000) in contrast to Arabidopsis. It would be interesting to test if the Arabidopsis Atbrc1 mutant has increased auxin transport as observed in max mutants (Bennett and Leyser, 2006), as this would reveal whether the relative pleiotropy of the SL deficient and SL response mutants in comparison to Atbrc1 is related to a difference in PAT.

In Arabidopsis two TB1 homologues have been identified, AtBRC1 and AtBRC2 with a role in the control of shoot branching (Aguilar-Martinez et al., 2007). The presence of a second BRC1 homologue in pea and gene redundancy could also explain the weaker phenotype of Psbrc1 in comparison to the SL mutants. Nevertheless we were unable to amplify another pea BRC homologue, and in Arabidopsis the double Atbrc1 Atbrc2 mutant displays the same branching phenotype as the strongest Atbrc1 mutant (Aguilar-Martinez et al., 2007).

PsBRC1 acts downstream of the SL pathways to control axillary bud outgrowth

The fact that the Psbrc1 mutant did not respond to SL application suggests that PsBRC1 may be involved in the SL signaling pathway to repress axillary bud outgrowth. In support of BRC1 acting in the SL pathway, we showed that PsBRC1 expression in axillary buds was rapidly enhanced by SL treatment. PsBRC1 transcript levels were very low in axillary buds of SL deficient (rms1) or SL response (rms4) mutants in comparison to those in WT but were rapidly up-regulated by SL application particularly in rms1 in which it was already very low and in WT, rms2 and Psbrc1. As expected for a SL response mutant, PsBRC1 transcript levels remained very low after SL treatment in buds of the rms4 response mutant. Although RMS4 has not been proven to act in the SL pathway, it has all the features expected of a protein involved in SL signalling (branching phenotype of rms4, F-box protein,
non-response to GR24 application of the *rms4* mutant, strong feedback upregulating *RMS1* expression in the *rms4* mutant). As such, the *RMS*-dependent response of *PsBRC1* to SL is consistent with *PsBRC1* acting in the SL signaling pathway for branching inhibition. The expression of *PsBRC1* in the *Psbrc1* mutant was similar to its expression in WT and the increase of *PsBRC1* expression after GR24 application was not affected in the *Psbrc1* mutant despite the fact that the branching of this mutant was not repressed by GR24 application. These data indicate that *PsBRC1* transcript levels do not simply correlate negatively with the activity of axillary buds and suggest a direct effect of the *PsBRC1* transcription factor in repression of axillary bud outgrowth. In rice, the homolog of the maize *TB1* gene, *FC1* was found not to be transcriptionally up-regulated by SL (Minakuchi et al., 2010). In Arabidopsis, expression of *AtBRC1* was strongly down-regulated in the *max1* to *max4* mutants (Aguilar-Martinez et al., 2007) suggesting that SL up-regulates transcript levels of *AtBRC1* in Arabidopsis as we observed for *PsBRC1*. In contrast, the other homolog, *AtBRC2*, is not down-regulated in the *max1* and *max2* mutants; *FC1* and *AtBRC2* appear to be similarly unresponsive to SL while *AtBRC1* and *PsBRC1* are both transcriptionally up-regulated by SL.

To confirm that *PsBRC1* and *RMS1* act in the same pathway, *rms1 Psbrc1* double mutant plants were produced and their phenotype analyzed alongside the single mutant plants in a comparable genetic background. In Arabidopsis and rice, the corresponding double mutants have the same level of branching as single mutants (Aguilar-Martinez et al., 2007) suggesting that the FC1/AtBRC1 TCP transcription factors act in the SL pathway. In pea, surprisingly, total branch length was strongly enhanced in the *rms1 Psbrc1* double mutant plants mainly because of strong development of cotyledonary branches. When branching was quantified according the position along the stem, branching of *rms1 Psbrc1* was similar to branching in *rms1* at upper nodes (node 3 and above) but transgressive at basal nodes (cotyledonary node and node 1). These results suggest a possible specific regulation of branching at basal nodes in pea. Unlike in Arabidopsis, these buds are differentiated very early in the embryo at the axil of particular leaves (cotyledons and scale leaves) and their development generally occurs below the soil surface. In pea, the *RMS6* gene has been shown to control bud outgrowth only at these nodes (Rameau et al., 2002). Future studies will have to decipher how branching is regulated at these basal nodes and how *RMS6*, SL and *PsBRC1* interact to have a better understanding of the phenotype of the *rms1 Psbrc1* double mutant.
Our results showed that *PsBRC1* transcript levels decrease after direct application of CK to the axillary bud for all genotypes tested including the SL response *rms4* and *Psbrc1* mutants. These results demonstrate that CK controls *PsBRC1* expression independently of the SL pathway. Thus *PsBRC1* could integrate the SL and CK pathways at the transcriptional level within the bud and bud outgrowth would occur where *PsBRC1* falls below a certain transcript level. In rice, similar results have been obtained with a strong decrease of *FC1* expression 3 hours after BAP treatment (Minakuchi et al., 2010).

We tested the phenotypic response of CK application on axillary bud at node 4 of *Psbrc1Cam*, *rms4* and their corresponding WT progenitors. CK treatment induced bud outgrowth for all genotypes, including *Psbrc1Cam*. This response indicates that CK may promote axillary bud outgrowth and/or growth independently from the SL signaling pathway and from *PsBRC1*. The phenotypic response to decapitation was consistent with the CK response of *Psbrc1Cam* and of the SL deficient *rms1* mutant. This result differs from that observed in Arabidopsis where *Atbrc1* mutants showed no response to decapitation (Aguilar-Martinez et al., 2007). This difference between pea and Arabidopsis could be explained by the stage of development when the decapitation is performed (a floral shoot is decapitated in the case of Arabidopsis compared to decapitation of a vegetative shoot in pea) and by the different growth habits of these species (Cline, 1996). If *PsBRC1* integrates the SL and CK signals to control axillary bud outgrowth, the CK response of the *Psbrc1Cam* mutant could be interpreted as the CK response having at least two components, one involving early bud outgrowth (*PsBRC1*-dependent) and the other one involving sustained growth (*PsBRC1*-independent) (Dun et al., 2009). Indeed, branching involves many steps from axillary meristem initiation and axillary bud formation to axillary bud outgrowth and sustained growth of branches. Simple quantification of the length of buds/lateral branches does not in itself distinguish the different growth stages.

The RMS2-dependent feedback signal originates between RMS4 and PsBRC1

The existence of a mobile graft-transmissible auxin-independent shoot-to-root feedback-signal that up-regulates *RMS1* and *RMS5* transcription and down-regulates X-CK export from roots was previously proposed (Beveridge et al., 2009; Dun et al., 2009). Because the *rms2*
mutant showed low RMS1 expression and high X-CK content in the xylem sap, in contrast to the other rms mutants, it was suggested that the shoot-to-root feedback signal was RMS2-dependent (Beveridge et al., 2000). Experiments with grafted plants bearing two shoots of different genotypes and different phenotypes indicated that the feedback signal was more likely generated by the branching shoot to suppress X-CK and up-regulate RMS1 expression even in the presence of a non branching WT shoot. Moreover, experiments with mutants lacking axillary meristems in an rms4 background demonstrated that branching per se was not the cause of the feedback (Foo et al., 2001; Foo et al., 2007). It was instead suggested to be regulated by the absence of perception of SL (Foo et al., 2005; Dun et al., 2009), as various mutants unable to produce (rms1, rms5) or to respond (rms3, rms4) to SL have reduced export of X-CK from the roots (Beveridge et al., 1997b; Morris et al., 2001; Foo et al., 2007) and increased expression of SL biosynthesis genes (Foo et al., 2005; Johnson et al., 2006; Arite et al., 2007; Hayward et al., 2009). Here we showed that Psbrc1Cam lacks the feedback upregulation of RMS1 gene expression and that the substantial down-regulation of X-CK content in SL biosynthesis and signaling mutants was not found in Psbrc1Cam plants (Table 1). Instead, X-CK was either near-normal in Psbrc1Cam or substantially increased in Psbrc1Te mutant plants, with the response appearing to depend on genetic background in our experiments. Similarly in rice, D10 expression is not increased in the fc1 mutant whereas it is highly expressed in other SL-related mutants (Arite et al., 2007; Minakuchi et al., 2010). As discussed above, our results suggest that PsBRC1 acts downstream of RMS4 in the SL signaling pathway. The absence of feedback in Psbrc1 could suggest that the SL-mediated feedback regulation is generated between the action of RMS4 and PsBRC1. This is further supported by the feedback down-regulation of X-CK in Psbrc1 rms1 double and rms1 single mutants but not in Psbrc1Te single mutants.

Advances in plant hormone signaling have highlighted the role of the ubiquitin-proteasome pathway and targeted protein turnover (Santner and Estelle, 2009). Because the MAX2/RMS4 gene encodes an F-box protein (Stirnberg et al., 2002; Johnson et al., 2006), it is likely that the SL signaling pathway involves similar targeted protein degradation. In the gibberellin (GA) signaling pathway, DELLA proteins are repressors that act directly downstream of GA receptor. Microarray analysis has identified early GA and DELLA responsive genes (Zentella et al., 2007). Among the GA-repressed and DELLA-induced targets are genes encoding GA biosynthetic enzymes, indicating direct involvement of DELLA proteins in feedback regulation. Proteins targeted for degradation in the SL signaling pathway, still to be identified,
and possibly acting as repressor of \( \text{PsBRC1} \) may similarly regulate the feedback signal in pea controlling SL biosynthesis and X-CK.

SL quantifications in root exudates of \( \text{Psbrc1} \) genotypes in comparison to their WT show that this mutant is not deficient in SL biosynthesis and explains the grafting results. The higher level of fabacyl acetate found in root exudates of the two \( \text{Psbrc1} \) lines in comparison to their WT, despite low \( \text{RMS1} \) expression in epicotyl, is surprising and indicates possible mis-regulation of SL biosynthesis in the mutant. In the rice \( \text{fc1} \) mutant, level of epi-5DS in root exudates was slightly higher but not significantly different from WT (Minakuchi et al., 2010). (Dun et al., 2009) using an hypothesis-driven modeling approach, suggested that SL biosynthesis is tightly regulated by multiple feedback signals in both shoot and root. More experimental data, e.g. \( \text{RMS1} \) expression in \( \text{Psbrc1} \) roots, SL quantifications in shoot, xylem and root, are needed to have a complete understanding of the signal network controlling shoot branching.

**CONCLUSION**

This study shows that the \( \text{PsBRC1} \) transcription factor acts locally in the axillary bud and strongly supports that it acts downstream of SL to repress bud outgrowth. Two models are currently proposed to explain how auxin and SLs interact to control branching: the auxin transport canalization model and the second messenger model (Brewer et al., 2009; Domagalska and Leyser, 2011). The precise comparison of the \( \text{Psbrc1}^{Te} \) mutant phenotype with the SL synthesis (\( \text{rms1} \)) mutant suggests that processes within the stem may be affected in SL synthesis mutants but not in \( \text{Psbrc1}^{Te} \), one of these processes being PAT. The model that best fits with our data would be one in which both regulatory systems, not mutually exclusive as indicated in (Domagalska and Leyser, 2011), would co-exist (Fig. 6). The degree of branching of a plant is an important component of its fitness and it is very likely that different pathways are involved in its tight control according to bud position on the stem, developmental stage and environmental conditions. SL would act locally in axillary buds via \( \text{PsBRC1} \) and would also coordinate branching across the plant (Leyser, 2011) by controlling auxin transport independently from \( \text{PsBRC1} \). The study of \( \text{PsBRC1} \) gives an example of a transcription factor with more limited phenotypic effects than its upstream signaling genes. It indicates that the SL signaling pathway may be shared among multiple developmental modules as proposed by (Doebley and Lukens, 1998). Future studies will decipher these different components of SL function.
Materials and methods

Isolation of PsBRC1, phylogenetic analysis and mapping.

Two degenerate primers, Cyc5F (5’-GGGA(T/C)CG(G/A)(T/C)(G/T/C)TC-3’) and Cyc5R (5’-CTT(G/T)(T/C)TCTT(G/T)(T/C)(T/C)CT-3’) were designed in conserved TCP domain of *Lotus japonicus CYCLOIDEA5* (*LjCYC5*) gene. These primers were tested on pea (*Pisum sativum*) Térèse cultivar genomic DNA and a fragment of 400 bp was amplified. The 5’ region was obtained with one round of 5’RACE PCR using gene specific nested primers 642Dn (5’-CCACTTTTTCTTGTTCTTGGATTT-3’), 527Dn (5’-TGTTTGATTCCGTCTTTCG-3’), and 497Dn (5’-TAACCAGTCCACAGTTTTC-3’), followed by a PCR walking with 3 specific primers 497Dn, 198R (5’-GCTAGATCTTTTCCTTTGGATC-3’), and 143R (5’GCTTCTGCAGGAACAGAC-3’), and the restriction enzyme *Dra*I (Fermentas). The 3’ sequence of PsBRC1 was obtained by TAIL PCR (Liu and Whittier, 1995) with gene specific nested primers 198F (5’-GATCCAAAGGAAAAGATCTAGC-3’), and 527Un (5’-CGAAAGACGGGAATCAAAC-3’) and the RAPD primer E1(5’-CCCAAGGTCC-3’). The amplicon obtained was sequenced using 642Un (5’-AAATCAAGAACAAGAAAAATGG-3’) and allowed to identify a 1100 bp sequence. The full 1576 bp sequence contains 22 bp 5’ UTR and 187 bp of the 3’ UTR.

Subsequent mapping was realised in the Recombinant Inbred Line population (Térèse x K586) (Laucou et al., 1998) using a CAPS marker. The amplification was realised with primers 198F and 527Dn and the product digested using *Sfe*I (Fermentas), cutting the sequence corresponding to the peptidic CTRYAG sequence only in the Torsdag/K586 genotype.

Obtention of Psbrc1 mutant by TILLING screening.

Psbrcl mutants were identified from an EMS population containing 4800 *Pisum sativum* lines using TILLING screening (McCallum et al., 2000). PCR and digestion were performed as described in (Dalmais et al., 2008). A 1 Kb first amplicon was amplified with primers PsCycN1F 5’-GTCTTGTTCCTGCAGAAGC-3’ and PsCycN1R 5’-GTGCAAGTACATGGATGG-3’ with an annealing temperature of 60°C. From this
one, a second amplicon of 900 bp was amplified with primers PsCycN2Ftag 5′-ACGACGTTGTTAAAACGACCTGCTTCTGGTAAAGGC-3′ and PsCycN2Rtag2 5′-ATAACAATTTCACACAGGTTTTCCAAGGACTCGTG-3′ with an annealing temperature of 58°C. The gene-specific inner primers carried a universal M13 tail (underlined). To confirm mutations, PCR products were sequenced (GATC Biotech, Germany) and sequence analysis was performed (Chromas v.1.4523 software). PsBRC1 partial genomic sequence and TILLING mutations were integrated in UTILLdb (http://urgv.evry.inra.fr/UTILLdb).

Mutation of the 4654 family was followed using CAPS marker amplified with primers 4654-8F (5′-GTCTTGTTTCTCAGAGCTG-3′) and 4654-317R (5′-CCAAGCTTGAAACTCCTTC-3′) and digested with TasI enzyme (Fermentas).

Plant materials, growing conditions and phenotype measurement.

Plants used in this study derived from various cultivars of pea (Pisum sativum). The rms1-10 (M3T-884) and rms4-3 (M3T-946) mutants were obtained in Térèse cultivar (Rameau et al., 1997). The rms2-1 allele obtained in Torsdag (Arumingtyas et al., 1992; Beveridge et al., 1997a) was backcrossed in Térèse background and the BC7 (Térèse x K524) was used in this study. The Psbrc1 mutant families derived from the TILLING approach were obtained from the cultivar Caméor (Dalmais et al., 2008).

The Psbrc1 mutant was first back-crossed with its WT progenitor Caméor. For the segregation analysis, a BC2-F2 (4654 x Caméor) population was genotyped for the T195I mutation with a CAPS marker (see below) and was phenotyped when plants had 10 leaves expanded. A BC3 mutant line (Psbrc1) was used for further study and named Psbrc1Cam. To have a better comparison with the rms1 mutant used in the study (line M3T-884), the Psbrc1 mutant was also back-crossed twice in the line Térèse (BC2 (Té x Psbrc1)) and this line was named Psbrc1Té. To analyze the phenotype of rms1 Psbrc1 double mutant plants and compare it to the branching phenotype of single mutants in a comparable genetic background, a cross between M3T-884 (rms1) and an F2 (Térèse x Psbrc1) plant that was Psbrc1 and afila (without leaflets as Térèse) was done. More than 100 F2 plants were genotyped and phenotyped for RMS1 and PsBRC1 and several F3 families were also analysed for their branching phenotype.

For CK quantifications in sap of double mutant, 28 rms1, 27 Psbrc1, 40 rms1 Psbrc1 plants were selected in the F3 generation derived from 4 F2 (M3T-884 x F2 (Té x Psbrc1Cam)) plants; these F2 plants were fixed for one mutation and heterozygous for the other one and gave in F3 generation ¼ single mutant, ¼ double mutant plants and ½ plants with the same
genotype as the parental plant (sap has not been collected from the later plants). Approximately 30 WT plants from 2 F2 WT plants for both genes were also used as well as the WT Térèse, the rms1 mutant (M3T-884) and the Psbrc1Té line.

For genotyping the T195I mutation in PsBRC1 in the different crosses, a CAPS marker was designed. The amplified PCR fragment using primers 4654-8F (5’-GTCTTGTTCTGCAGAAGCTG-3’) and 4654-317R (5’-CCAAGCTTGAAACTCCTTCAC-3’) was digested with TaqI enzyme (Fermentas). The CAPS marker for genotyping RMS1 was based on the amplification of the PCR fragment using primers RMS1-118F (5’-TTGGTTGACCTCAGCTTGG-3’) and RMS1-984R (5’-CACAACAATGCAATGACAGC-3’) and the digestion with the Cfr13I enzyme (Fermentas).

Plants were grown in pots filled with taube, peat and soil (1:1:1), supplied regularly with nutrient solution in a heated glasshouse (15°C night and 22°C day) under a 16 hour photoperiod (the natural day length was extended or supplemented during the day when necessary using sodium lamps). For harvesting axillary buds at node 4 or epicotyls, plants were sown individually in 4 dL pots. For longer culture, 1 or 2 plants were cultivated per 2 L pots.

Nodes were numbered acropetally from the first scale leaf as node 1. The stage indicated corresponds to the number of nodes with fully expanded leaves.

Hormonal treatment.

The synthetic strigolactone, GR24 (kindly provided by F.D. Boyer, Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France) was applied on buds as 10 µL of a solution containing 50% of ethanol, 1% of polyethylene glycol 1450 (Sigma), 0.1% of dimethyl sulfoxide (DMSO) (Sigma) and 0.1% of acetone containing or not (mock treated) 500 nM of GR24.

Cytokinin, 6-benzyl aminopurine (BAP) (Sigma) was applied to buds as 10 µL of a solution containing 50% of ethanol, 1% of polyethylene glycol 1450 (Sigma) and 0.5% of dimethyl sulfoxide (DMSO) (Sigma) containing or not (mock treated) 50 µM of BAP.

Gene expression analysis.

RNA extraction and cDNA synthesis.

Tissue samples were harvested and grounded into liquid nitrogen. Total RNA was isolated from 20 to 30 buds or 10 to 15 epicotyls using TRIZOL reagent (Invitrogen) following the
manufacturer protocol. DNAse treatment was performed using Qiagen RNase-Free DNase Set (79254) and RNeasy Mini Kit (74904) and eluted in 50 µL of RNase free water. RNA was quantified using NanoDrop 1000 and migrated on gel to check RNA non degradation. Absence of contamination with genomic DNA was checked using 35 cycles PCR with RMS1 primers (see below).

Total cDNA was synthesised from 2 µg of total RNA using 50U of RevertAid H Minus M-MLV Reverse Transcriptase (Fermentas) in 30 µL following the manufacturer instructions with poly T (18) primer. cDNA was diluted 10 times before subsequent analysis.

Real-Time PCR analysis.

Quantitative RT-PCR analyses were performed using SYBR ROX RealMasterMix (5Prime) with specific primers:

**PsBRC1**: BRC1 forward : 5’ AGGCAAGAGAAAGAGCAAGG 3’, BRC1 reverse : 5’ TTGCATTGCTTTGAGTGGTA 3’. (amplicon of 128 bp)

**RMS1**: RMS1 forward 5’TTGCTCAGGGCTGAACCAAC 3’, RMS1 reverse : 5’ CACTTCCACACTTGCCACAATC 3’. (amplicon of 113 bp)

Cycling conditions for amplification were 95°C for 10 min, 50 cycles of 95°C for 5 s, 62°C for 5 s, and 72°C for 15 s followed by 0.1°C·s⁻¹ ramping up to 95°C for fusion curve characterization. Three biological repeats were analysed in duplicate. To calculate relative transcript levels, the comparative cycle method based on non-equal efficiencies was used (Pfaffl, 2001). Transcript levels for the different genes were expressed relative to the expression of **EF1α** gene (Johnson et al., 2006).

Cytokinin quantification

Xylem sap was harvested from roots of 30 day old plants by applying vacuum to the freshly cut epicotyl with a syringe. Independent pools of 3 mL of sap were used to quantify cytokinins as described by (Morris et al., 2001).

Strigolactone sampling and analysis
Pea plants were germinated in vermiculite for 6 d, then transferred to aerated hydroponic complete nutrient solution culture, with 12 plants in 6 litres of solution in a growth cabinet set at 23°C 55% RH day and 15°C 65% RH night, with a 16 h photoperiod, and light intensity of 300 µmol m⁻² s⁻¹ provided by cool white fluorescent lamps, supplemented with tungsten lamps. At 19 d after germination, the solution was replaced with water, then 24 h later batches of 12 plants were transferred to 1 litre 900 ml of water into which exudate was collected for 24 h. d-labelled strigolactone internal standards (20 ng each of d₁-orobanchol, d₁-orobanchyl acetate, d₁-epi-orobanchyl acetate, d₁-fabacyl acetate, d₆-5-deoxystrigol and d₆-epi-5-deoxystrigol, all generous gifts of Koichi Yoneyama) were added to each sample. Strigolactones were extracted with 0.6 volume of ethyl acetate, followed by back extraction with 0.1 M KH₂PO₄. The ethyl acetate fraction was dried with anhydrous MgSO₄, filtered and evaporated to dryness at 35°C. Samples were redissolved in dry acetone, transferred to autosampler vials then re-dried dissolved in acetonitrile/water (30:70, v/v) and filtered (0.45 µm) prior to analysis by LC-MS-multiple reaction monitoring (MRM) in positive ion electrospray mode using an Agilent 1100 LC system and an Applied Biosystems Sciex QTrap mass spectrometer. Column was Phenomenex 3 µm C18 Luna 100 x 2 mm, heated to 40°C with a flow rate of 200 µl min⁻¹. Initial mobile phase was 45.5% acetonitrile in 0.1% aqueous formic acid. After 1 minute a linear gradient to 77% acetonitrile over 19 min was applied, then increased to 95% acetonitrile for 3 min. Appropriate MRM transitions were monitored for each labelled standard and corresponding unlabelled strigolactone. For quantitations reported here, the transitions were m/z 406-232 for d₁-fabacyl acetate and 405-231 for fabacyl acetate. Two biological replicates representing pools of 12 plants were analysed for each genotype. Strigolactone content was calculated from MRM peak areas by the stable isotope ratio method.

Statistical analysis
For the statistical analysis Student’s t-test and ANOVA were performed using Statgraphics Plus 5.1. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

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Figure Legends

Figure 1. Structure of *PsBRC1* gene and phenotype of corresponding mutant. A, Gene structure of *Pisum sativum BRANCHED1 (PsBRC1)* and location of mutations. Bases are numbered from start codon. TCP domain (from bp 441 to bp 630) is shown in red and corresponding protein sequence indicated below. Point mutations are indicated by triangles (black and red for the one studied here), boxes correspond to exons, blue area corresponds to the TILLed sequence. B, Comparison of WT Térèse plant (left) with rms4 mutant (middle left), WT Caméor plant (middle right) and *Psbrc1Cam* mutant plant (right). C, Branch length at each node of WT Caméor, *Psbrc1Cam* mutant, WT Térèse, *rms1* mutant and *Psbrc1Te*. Data are means +/- SE (n= 12). D, Means of the number of cotyledonary branches per individual observed in a segregating F2 population of 103 plants between M3T-884 (*rms1*) and a *Psbrc1* F2 plant (*Psbrc1Cam* x Térèse). n = number of plants observed per genotypic classes; ncot = number of plants with at least one cotyledonary branch. Data are means +/- SE (n= 12).

Figure 2. A, Effect of GR24 application on bud growth. Bud length at node 3 of WT Térèse (black), *rms1* (white), WT Caméor (grey), *Psbrc1Cam* (hatched) measured 10 d after treatment applied to bud of stage 5 intact plants with solution containing 0 or 500 nM GR24. Data are means +/- SE (n=12). B, Strigolactone levels in root exudates of WT and *Psbrc1* mutant plants. Exudates were collected into water for 24 h from 20 d-old hydroponically grown plants, and strigolactones were quantified by LC-MS using MRM transitions m/z 405-231 for fabacyl acetate and 406-232 for the d1-fabacyl acetate internal standard. Data are means +/- SE, based on analysis of two independent pools of 12 plants for each genotype.

Figure 3. Effect of GR24 on *PsBRC1* transcript levels. *PsBRC1* transcript levels relative to EF1α in axillary bud at node 4 after GR24 applications (white) or mock treated (black). RNA was extracted from dissected buds from pools of 30 plants at the six-node stage and quantified by real-time PCR. The data are representative of 2 to 3 independent experiments. A, at 6 h and 24 h after GR24 application of WT Térèse, *rms1*, *rms2* and *rms4* plants. Branching phenotype at node 4 after GR24 application is given below for each genotype. B, at 6 h and 48h of WT Térèse, *rms1*, *rms4* and *Psbrc1Te* plants. Data are means +/- SE (n=3).
Figure 4. Effect of BAP on PsBRC1 transcript levels and on bud growth. A, PsBRC1 transcript levels relative to EF1α in axillary bud at node 4 after BAP (50 µM) applications in WT Caméor, Psbrc1Cam, WT Térèse, rms1 and rms4. RNA was extracted from the dissected buds of 30 plants at the six-node stage and quantified by real-time PCR. The data are representative of 3 independent experiments; B, Effect of BAP (50 µM) treatment on bud growth at node 4 in WT Térèse, rms4, WT Caméor and Psbrc1Cam. Measures are done 5 days after treatment. Data are means +/- SE (n=12); C, Effect of decapitation above node 5 on total branch length at nodes 1 to 5. Intact plant and decapitated plant of Psbrc1Cam, WT Caméor, rms1, WT Térèse. Data are means +/- SE (n=8).

Figure 5. The RMS2-dependent feedback signal is not activated in Psbrc1. A, RMS1 transcript levels in epicotyls of rms1, rms2, rms4 and their corresponding WT Térèse and Psbrc1Cam and its corresponding WT Caméor. RNA was extracted from plant at stage 6. The data are representative of 3 independent experiments; B and C, Xylem cytokinin content of root xylem sap. tZ stands for trans-zeatin, DZ for dihydrozeatin, tZR for trans-zeatin riboside, DZR for dihydrozeatin riboside, cZR for cis-zeatin riboside, IP for isopentenyl adenine and IPR for isopentenyl adenosine. B, from WT Caméor and Psbrc1Cam mutant. C, from WT Térèse, M3T-884 (rms1), Psbrc1Té and from F3 plants with WT, rms1, Psbrc1, and rms1 Psbrc1 genotypes derived from 4 F2 (M3T-884 x F2 (Té x Psbrc1Cam)) (see Materials and Methods). Measurements were made from pools of 3 ml of sap harvested from 20 to 40 plants. Data are means +/- SE (n=3)

Figure 6. Model for the hormonal control of branching in pea integrating the function of PsBRC1 in the axillary bud and the auxin transport canalisation-based model (Domagalska & Leyser 2011). PsBRC1 integrates the SL and CK pathways to control bud outgrowth. CK also increase bud growth via a PsBRC1-independent pathway. Auxin maintains RMS1 transcript levels, hence SL synthesis and down-regulates CK levels. The RMS2-dependent feedback,that upregulates SL biosynthesis and down-regulates xylem CK, is activated when there is a lack of SL signalling via RMS4, and may be independent of PsBRC1. SLs reduce PIN accumulation to the plasma membrane via RMS4 but independently of PsBRC1 and, by reducing the effectiveness of the canalisation feedback loop, enhance the competition between active buds.
Figure S1. Phylogenetic tree of representative TCP proteins from *Arabidopsis thaliana* (At), PsBRC1 from pea and members of other plant species, *Antirrhinum majus* (Am), *Lotus japonicus* (Lj), rice (Os), maize (Zm), *Sorghum bicolor* (Sb). Evolutionary relationships were analyzed using a selection of predicted amino acid sequences of the TCP domains aligned with the program CLUSTALX (Thompson et al., 1997). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and the optimal tree was generated. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Arabidopsis and rice TCP genes named after (Martin-Trillo and Cubas, 2010); LjCYC1, LjCYC2, LjCYC3, and LjCYC5 (GenBank accession nos DQ202475, DQ202476, DQ202477 and DQ202478).

Figure S2. Comparison of branching mutants’ plant architecture. A, Branching at node 1 plotted against Total lateral branch length for homozygote Psbrc1 mutant and WT segregants from a BC2-F2 (4654 x Caméor) population; B, Comparison of internode length between nodes 1 to 6 of WT Caméor, *Psbr*1Cam, WT Térèse, *rms*1 (M3T-884) and *Psbr*1Té. Data are means +/- SE (n= 12). C, Stem diameter at node 3 of WT Caméor, *Psbr*1Cam, WT Térèse, *rms*1 (M3T-884) plants. Data are means +/- SE (n= 12). D, Branching phenotype of different F3 families from the cross (M3T-884 x (Térèse x Psbrc1 Cam)); 8 individuals per family; means for 6 Psbrc1 families, 5 rms1 families and 6 rms1 Psbrc1 families.

Figure S3. Grafting experiment showing that *PsBRC1* acts in the shoot and that the *Psbr*1Cam mutant is not strigolactone deficient. Different combinations of grafts between scion and rootstock of 7d-old plants of WT Caméor, *rms*1 (M3T-884), and *Psbr*1Cam as indicated below. Total branch lengths from nodes 1 to 6 were measured 39 days after grafting. Data are means +/- SE (n= 12).

Figure S4. Independent experiments for real-time PCR. A, Effect of GR24 on *PsBRC1* transcript levels. *PsBRC1* transcript levels relative to *EF1α* in axillary bud at node 4 after GR24 applications or mock treated at 6 h and 24 h after treatment of WT Térèse, *rms*1, *rms*2 and *rms*4 plants. RNA was extracted from dissected buds from pools of 30 plants at the six-node stage and quantified by real-time PCR. B, Effect of BAP on *PsBRC1* transcript levels *PsBRC1* transcript levels relative to *EF1α* in axillary bud at node 4 after BAP (50 µM) applications in WT Caméor, *Psbr*1Cam, WT Térèse, *rms*1 and *rms*4. RNA was extracted from the dissected buds of 30 plants at the six-node stage and quantified by real-time PCR. C,
RMS1 transcript levels in epicotyls of rms1, rms2, rms4 and their corresponding WT Térèse and of Pbsrc1Cam and its corresponding WT Caméor. RNA was extracted from plant at stage 6.
Table 1. Summary of the physiological characteristics of the different pea branching mutants. “RMS1 expression” stands for RMS1 mRNA accumulation in epicotyl, “Response to GR24” relates to the inhibition effect of GR24 on bud outgrowth, “X-CK” corresponds to CK concentration in xylem sap.

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Supplemental Table T1: List of the mutations identified in the *PsBRC1* gene using TILLING.
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Supplemental Table T2: Relative expression of *PsBRC1* in different plant organs. Lateral bud expression has been used as reference and attributed a 100 value (N.D. stands for Not Detected).
Figure 1. Structure of PsBRC1 gene and phenotype of corresponding mutant. A, Gene structure of Pisum sativum BRANCHED1 (PsBRC1) and location of mutations. Bases are numbered from start codon. TCP domain (from bp 441 to bp 630) is shown in red and corresponding protein sequence indicated below. Point mutations are indicated by triangles (black and red for the one studied here), boxes correspond to exons, blue area corresponds to the TILLed sequence. B, Comparison of WT Térèse plant (left) with rms4 mutant (middle left), WT Caméor plant (middle right) and Psbrc1Cam mutant plant (right); C, Branch length at each node of WT Caméor, Psbrc1Cam mutant, WT Térèse, rms1 mutant and Psbrc1Te. Data are means +/- SE (n= 12). D, Means of the number of cotyledonary branches per individual observed in a segregating F2 population of 103 plants between M3T-884 (rms1) and a Psbrc1 F2 plant (Psbrc1Cam x Térèse). n = number of plants observed per genotypic classes; ncot = number of plants with at least one cotyledonary branch. Data are means +/- SE (n= 12).
Figure 2. A, Effect of GR24 application on bud growth. Bud length at node 3 of WT Térèse (black), rms1 (white), WT Caméor (grey), Psbrc1Cam (hatched) measured 10 d after treatment applied to bud of stage 5 intact plants with solution containing 0 or 500 nM GR24. Data are means +/- SE (n=12). B, Strigolactone levels in root exudates of WT and Psbrc1 mutant plants. Exudates were collected into water for 24 h from 20 d-old hydroponically grown plants, and strigolactones were quantified by LC-MS using MRM transitions m/z 405-231 for fabacyl acetate and 406-232 for the d1-fabacyl acetate internal standard. Data are means +/- SE, based on analysis of two independent pools of 12 plants for each genotype.
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