Strigolactone (SL), auxin, and cytokinin (CK) are hormones that interact to regulate shoot branching. For example, several ramosus (rms) branching mutants in pea (Pisum sativum) have SL defects, perturbed xylem CK levels, and diminished responses to auxin in shoot decapitation assays. In contrast with the last of these characteristics, we discovered that buds on isolated nodes (explants) of rms plants instead respond normally to auxin. We hypothesized that the presence or absence of attached roots would result in transcriptional and hormonal differences in buds and subtending stem tissues, and might underlie the differential auxin response. However, decapitated plants and explants both showed similar up-regulation of CK biosynthesis genes, increased CK levels, and down-regulation of auxin transport genes. Moreover, auxin application counteracted these trends, regardless of the effectiveness of auxin in inhibiting bud growth. Multivariate analysis revealed that stem transcript and CK changes were largely associated with decapitation and/or root removal and auxin response, whereas bud transcript profiles related more to SL defects. CK clustering profiles were indicative of additional zeatin-type CKs in decapitated stems being supplied by roots and thus promoting bud growth in SL-deficient genotypes even in the presence of added auxin. This difference in CK content may explain why rms buds on explants respond better to auxin than those on decapitated plants. We further conclude that rapid changes in CK status in stems are auxin dependent but largely SL independent, suggesting a model in which auxin and CK are dominant regulators of decapitation-induced branching, whereas SLs are more important in intact plants.
integrated through opposite actions on expression of the TCP transcription factor Teosinte Branched1 (TB1)/BRANCHED1 (BRC1) in buds (Minakuchi et al., 2010; Braun et al., 2012; Dun et al., 2012). In pea, CK is a negative regulator and SL is a positive regulator of PsBRC1 (Braun et al., 2012). By contrast, SL application did not change expression of maize (Zea mays) or rice TB1/BRC1 orthologs (Minakuchi et al., 2010; Guan et al., 2012). However, increased shoot branching in loss of function brc1 mutants of Arabidopsis, rice, and pea is not rescued by SL addition (Brewer et al., 2009; Minakuchi et al., 2010; Braun et al., 2012), indicating that SL signaling is dependent on BRC1. The consensus view is that TB1/BRC1 and related genes may be conserved branching regulators.

SL also influences CK levels, notably the greatly reduced xylem sap CK content of most SL-defective mutants (Beveridge et al., 1994, 1997a, 1997b; Morris et al., 2001; Foo et al., 2007). However, the pea mutant rms2 is an exception, displaying increased xylem CK, associated with low SL biosynthesis gene expression (Beveridge et al., 1997b, Foo et al., 2005). Although isolation of the RMS2 gene has not been reported, it is proposed to function in a feedback loop that regulates both root CK export and SL biosynthesis (Dun et al., 2009). At the biosynthetic level, expression of the CK biosynthesis gene *Pisum sativum ISOPENTENYL TRANSFERASE1* (PsIPT1 [but not PsIPT2]) is increased in SL mutants, although addition of synthetic SL to isolated nodes did not affect transcript levels for either IPT gene (Dun et al., 2012).

SL mutants show greatly reduced response to auxin in inhibiting shoot branching of decapitated pea plants (Beveridge et al., 2000) and isolated nodes of Arabidopsis (Sorefan et al., 2003). Two nonexclusive models have been advanced to account for the regulation of bud outgrowth. The first, a variant on classic auxin canalization concepts (Sachs, 1968, 1981), proposes that bud dormancy is sustained through a failure to develop a polar transport stream exporting auxin from the bud, due to competition for finite auxin transport capacity in the stem, with the flow from through the main shoot being dominant over that from buds (Sachs, 1970; Bennett et al., 2006; Domagalska and Leyser, 2011; Müller and Leyser, 2011). Shoot tip removal depletes stem auxin pools, diminishes competition for the auxin transport system, and thus permits establishment of auxin export from buds. Altered PIN-FORMED (PIN) auxin transporter expression and/or localization in response to added SL (Shinohara et al., 2013) and in SL mutants (Bennett et al., 2006; Cazzonelli et al., 2009; Hayward et al., 2009; Crawford et al., 2010; Balla et al., 2011; Ruyter-Spira et al., 2011) may explain the enhanced auxin transport in SL mutants (Beveridge et al., 2000; Bennett et al., 2006; Crawford et al., 2010). Greater overall transport capacity in SL mutant stems then allows bud auxin export. Counterevidence shows that shoot tissues can have very high auxin transport capacity, well beyond that required to carry quantities of auxin found in nature (Brewer et al., 2009; Renton et al., 2012), leading to low competition for auxin transporter binding sites.

Moreover, chemical inhibition of auxin transport in stem tissue above a bud does not necessarily stimulate that bud to grow (Morris et al., 2005; Ferguson and Beveridge, 2009). Instead, an alternative model depends largely on the regulatory relationships among the different hormones (Ferguson and Beveridge, 2009; Beveridge and Kyozuka, 2010). Auxin induces expression of SL biosynthesis genes (Sorefan et al., 2003; Bainbridge et al., 2005; Foo et al., 2005; Ishikawa et al., 2005; Johnson et al., 2006; Arite et al., 2009; Hayward et al., 2009), but represses CK biosynthesis in the shoot (Tanaka et al., 2006; Dun et al., 2012). The combined effect of increased SL and reduced CK is proposed to lead to bud growth inhibition. A recent additional discovery is that sugars, rather than auxin, are necessary and sufficient to regulate the very earliest stages of bud outgrowth following decapitation (Mason et al., 2014). The demand for sugars by the intact shoot tip was shown to override the effects of auxin depletion by preventing the initial outgrowth of axillary buds.

The two branching models described above provide reference points for this study, which focuses on tissue-specific regulation of hormone status and expression of hormone-related genes under defined conditions that affect shoot branching. Because SL has multiple influences on shoot branching, auxin response, and xylem CK levels, we were particularly interested to test the SL dependence of auxin responses at molecular and phenotypic levels. Because roots contribute to the CK pool in the shoot, we predicted that presence or absence of roots should have a significant impact on CK levels and expression of hormone-related genes in shoot tissues. We further hypothesized that spatial regulation would be a significant factor, and we thus separately analyzed transcript and hormone levels from bud and subtending nodal stem tissues. Pea was used as an ideal physiological test system, where genetic (SL mutant), chemical (auxin application), and physical (decapitation and node isolation) approaches were applied to create a series of conditions in which bud growth would be activated or suppressed. Using multivariate statistical analysis, we show here both SL-dependent and SL-independent processes, highly divergent tissue-specific regulation of transcript and hormone levels, and coordinate expression of CK and auxin genes in different clusters.

**RESULTS**

Conditional Auxin Response in SL Mutants

Previous research showed that decapitated rms plants are unresponsive to exogenous auxin supplied to the apical end of decapitated shoots of whole plants (Beveridge et al., 2000), even at doses that vastly exceed shoot endogenous auxin content (Foo et al., 2005), but the underlying explanation remains unclear. Because grafting can influence branching of several rms mutants and regulatory signals such as CKs and SLs can originate in roots, we undertook experiments to
SL-Independent Changes in Auxin Transport Gene Expression in Stems

To test for the possible molecular basis of the conditional regain of auxin response in explants of *rms* mutants, expression of auxin efflux carrier (pea *PIN1* [PsPIN1] and PsPIN2), auxin influx carrier (pea *AUXIN TRANSPORTER PROTEIN1* [PsAUX1]), and *PROTEIN KINASE2* (PK2, the pea ortholog of Arabidopsis *PINOID*; Bai et al., 2005) genes in stem and bud tissues were measured (Fig. 2; Supplemental Fig. S1). *PsPIN1* is in the same clade as Arabidopsis *PIN1* (AtPIN1) and, although not necessarily its ortholog, appears to cross react with AtPIN1 antibodies. *PsPIN2* is in the *AtPIN3/4/7* clade (Schnabel and Frugoli, 2004). Both *PsPIN* genes were previously reported to be expressed in shoot tissues as has *PsAUX1*, which is the likely ortholog of Arabidopsis *AUX1* (Schnabel and Frugoli, 2004). We sampled up to 6 h after treatment, which is sufficient time for bud growth activation (Morris et al., 2005). From typical responses in other reports (Balla et al., 2011; Waters et al., 2012), we predicted that removal of apical auxin sources would lead to rapid down-regulation of auxin transporter gene expression in stems, but this should be reversed by replacement with an exogenous auxin supply.

In most cases, decapitation-induced declines in *PsPIN* gene expression in the stem were similar in explants and decapitated plants, and were prevented or reversed by auxin application. Six h after decapitation or node isolation, *PsPIN1* transcript levels in wild-type and *rms1* stems had fallen dramatically (4- to 12-fold, *P < 0.01*; Fig. 2A). In *rms2*, no significant reductions were detected, although expression of both *PIN* genes in intact plants was already lower (2- to 4-fold, *P < 0.05*) than in the other genotypes. In all genotypes, auxin application resulted in higher *PsPIN1* expression (3- to 15-fold; *P < 0.01*) than in corresponding control decapitation and explant treatments. Changes in *PsPIN2* expression were smaller than for *PsPIN1* (Supplemental Fig. S1A). There was little evidence that the *rms1* mutation blocked reductions in stem *PsPIN1* transcript levels following decapitation or node isolation, and neither *rms* mutation prevented auxin from increasing *PIN* expression. This is supported by multivariate statistical analysis presented in Figure 3, in which all of the auxin transport genes clustered together as a functional group (loadings plot, Fig. 3A). Based on the directions of shifts between genotype × treatment combinations in the scores plot (Fig. 3B), expression of these genes is strongly associated with auxin response and is not greatly affected by SL deficiency, as depicted by alignments with overall factor responses in Figure 3C.

Trends for *PIN* transcripts in buds (Fig. 2C; Supplemental Fig. S1B) clustered separately from those in stems (Fig. 3). Bud *PsPIN1* transcript levels were relatively stable among genotypes and in decapitation treatments. One exception was that *PsPIN1* levels fell in buds on *rms* explants, with these changes being reversed by auxin.

**rms-Dependent Changes in *PsAUX1* and *PK2* Gene Expression in Buds

In contrast with the relatively stable *PsAUX1* expression in stem tissues (Fig. 2B), *PsAUX1* transcript levels in buds were greatly influenced by genotype but were minimally affected by any of the treatments. Specifically, *PsAUX1* transcript abundance across genotypes was consistently in the order of the wild type > *rms1* > *rms2*, with levels in *rms1* 3- to 8-fold lower (*P < 0.001*) than in the wild type, and 12- to 60-fold lower in *rms2* (*P < 0.001*; Fig. 2D). Genotype effects on patterns of *PK2* expression in buds approximately mirrored...
those for PsAUX1 (Supplemental Fig. S1D), with no response to decapitation or auxin application. However, unlike PsAUX1, PK2 expression changes in stems (Supplemental Fig. S1C) were similar to those of PsPIN1, with depletion following decapitation or node isolation, and recovery if auxin was supplied. Taken together, multivariate analysis confirms that a group of genes including PsAUX1 and PK2 cluster together (Fig. 3A), showing strong rms-dependent expression changes in buds, deduced from alignment with genotype responses shown in Figure 3B and summarized in Figure 3C. Importantly, these rms-dependent changes in gene expression in buds occurred across all treatments, and were therefore poorly correlated with bud outgrowth.

Decapitation or Node Isolation Causes Similar Rapid Declines in SL Biosynthesis Gene Expression That Are Reversed by Auxin

It was possible that the differential auxin response in decapitated rms stems versus explants reflected differing degrees of regulation and importance of SL (e.g. SL being the major regulator in decapitated plants but not in explants). We therefore examined expression of the auxin-responsive SL biosynthesis gene, RMS1, in stem samples. Transcripts were undetectable in rms1-1 tissues, as predicted for this deletion mutant, and were generally below detection limits in rms2, consistent with very low levels shown in previous studies (Foo et al., 2005). In wild-type stems, decapitated plants and explants both showed a rapid decline, around 10-fold after 1 h, and levels reduced even further by 6 h (Fig. 4). Where auxin was applied, most of the reduction in RMS1 expression still occurred by 1 h, but levels had stabilized or partially recovered by 6 h.

PsIPT Expression Is SL Independent in Stem Tissue, But Expression in Buds Is Altered in SL-Deficient Mutants

Because CKs are positive regulators of branching, we wished to establish whether local expression of CK biosynthesis genes in stems and buds was differentially affected by genotype and treatment. Comparative analysis of CK levels in tissues from decapitated plants and explants (see below) allowed indirect estimation of the likely contribution of roots and additional tissues of decapitated plants to shoot CK pools. Although SL mutants respond fully to exogenous CKs (Dun et al.,

<table>
<thead>
<tr>
<th>Plant</th>
<th>Intact Plant</th>
<th>Decapitated</th>
<th>Decapitated + Auxin</th>
<th>Explant</th>
<th>Explant + Auxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>rms1</td>
<td>(−)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>rms2</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

*Bud largely suppressed at this age in rms1, but typically grows out later.*
the contribution of endogenous CKs to the \textit{rms} branching phenotype remains unclear. Moreover, the mechanism by which levels of some CKs in xylem and shoot tissues are misregulated in \textit{rms} mutants (Foo et al., 2007) has yet to be established. Here, we examined levels of CKs and transcripts of \textit{PsIPT1} and \textit{PsIPT2}, the major CK biosynthesis genes expressed in pea shoots (Tanaka et al., 2006). As with the auxin transport genes, expression profiles of \textit{PsIPT} genes differed between stem and bud tissues, with stems having a comparatively stronger response to decapitation, node isolation, and auxin, and buds showing a significant SL (mutant) effect. Transcript levels of both \textit{PsIPT1} and \textit{PsIPT2} increased very substantially (10- to 100-fold; \(P < 0.001\)) in stem tissues of all genotypes by 6 h after node isolation or decapitation, and these increases were significantly suppressed by auxin addition (Fig. 5, A and B). Stem \textit{IPT2} expression increased more rapidly than \textit{IPT1}, within 1 h (Supplemental Fig. S2), and thus ahead of bud growth initiation.

In contrast with the similar patterns across genotypes in stems, \textit{IPT} expression in buds differed greatly between wild-type and \textit{rms} plants (Fig. 5, C and D) further emphasized by orthogonal relationships shown in Figure 6A. Transcript levels of both \textit{IPT} genes in intact plants were substantially lower in \textit{rms} mutants compared

![Figure 3](image-url)
with the wild type: 200- to 2000-fold less for \textit{PsIPT1}, and 50- to 200-fold less for \textit{PsIPT2} ($P_{0.001}$; Fig. 4, C and D). A second major difference in buds compared with stems was that no consistent increase in bud \textit{IPT} expression was seen in response to decapitation or node isolation; moreover, auxin application did not consistently suppress bud \textit{IPT} expression. Bud \textit{IPT} expression therefore bore little apparent relation to bud growth status (Table I).

**CK Content of Stem and Bud Tissues Is Highly Divergent, Affected by SL Deficiency, and Strongly Responsive to Decapitation, Node Isolation, and Auxin Supply**

A range of endogenous CKs was examined, covering the bioactive nucleobases, together with nucleoside (ribosyl), nucleotide (5' phosphoribosyl), and glucoside forms for each of the four major N6 isoprenoid side chain types, represented by the nucleobases transzeatin (tZ), cis-zeatin (cZ), dihydrozeatin (DZ), and isopentenyl adenine (IP; Supplemental Table S1). Of these, glucoside and cZ nucleobase levels were very low and inconsistently detected, and are not reported. Significant effects of genotype and treatment were found for total CK levels, with some differential effects on particular side chain classes and individual compounds.

In stem tissues of intact plants, \textit{rms} mutants had approximately 2-fold higher total CK content compared with wild-type tissues ($P_{0.05}$; Fig. 6A; Supplemental Fig. S3). In \textit{rms1}, the increase was largely due to elevated levels of IP-type CKs (around 5-fold), whereas in \textit{rms2} tissues, tZ compounds and additional phosphorylated CKs were elevated (Fig. 6; Supplemental Fig. S2). Six h after decapitation or node isolation, levels of many IP and tZ CKs showed significant increases in all genotypes, most notably zeatin riboside and isopentenyl adenosine (IPR) in decapitated plants (up to 10-fold, overall $P_{0.001}$) and IPR and isopentenyl adenosine-5'-phosphate (IPRP) in explants (up to 20-fold, overall $P_{0.001}$; Fig. 6; Supplemental Table S3). Auxin treatment negated much of the increases caused by decapitation and node isolation, with total CK levels being 3- to 10-fold lower ($P_{0.05}$) and restored close to intact levels. Multivariate analysis confirmed that the principal effect of decapitation and explant excision (cluster shifts in Fig. 7B) was an increase in all CKs, apart from cZ compounds and DZ, based on alignment with factor 1 in the loadings plot shown in Figure 7A. Especially in explants, auxin did not exactly reverse CK profiles back to levels in intact plants as shown in Figure 7C, which summarizes the shifts between clusters in Figure 7B. Plotting factors 2 and 3 unexpectedly revealed strong clustering of CK compounds.
Figure 6. CK content of stem and bud tissues of decapitated plants and nodal explants. A to G, Stem tissues. H to N, Bud tissues. A and H, Total CK content. B to D and I to K, IP-type compounds. E to G and L to N, tZ-type compounds. Analysis by liquid chromatography (LC)-mass spectrometry (MS)-multiple reaction monitoring (MRM). All treatments were...
in the stem according to each of the four \(N^6\)-side chain types: IP, tZ, cZ, and DZ (Fig. 7D). There were major underlying differences between decapitated plants and explants: \(tZ\) CK types predominated in decapitated stems, whereas IP CK types were more abundant in explants (Fig. 6). A notable differential auxin response between decapitated plants and explants was the stronger suppressive effect (up to 18-fold; overall \(P < 0.001\)) on \(tZ\)-type compounds in explants compared with decapitated plants (Fig. 6, E–G). These contrasts are further emphasized by the separated treatment clusters in the corresponding scores plot (Fig. 7E) and resultant inferred overall treatment effects (Fig. 7F).

In bud tissues, the main CKs were IP- and \(tZ\)-type compounds, with IPRP and zeatin riboside-5'-phosphate (tZRP) the most abundant forms (Fig. 6, H–N). By contrast, \(DZ\) and \(cZ\) compounds were generally below detection limits. In all treatments except decapitated wild-type plants, total CK levels increased (2- to 10-fold, \(P < 0.05\)) in buds following shoot decapitation or node isolation, with the greatest response in decapitated \(rms\) plants (Fig. 6H). Auxin addition resulted in depleted CKs in buds on explants of all genotypes, but had a minimal effect in buds of decapitated wild-type and \(rms\) plants, pointing to a further underlying CK effect differentiating decapitated plants from explants. Multivariate analysis of combined stem and bud data (Supplemental Fig. S4) indicated that most bud CKs clustered together, indicating strongly correlated overall effects of genotype and treatment, but clearly differentiated from the corresponding compounds in stems.

**DISCUSSION**

**Auxin Inhibition of Bud Outgrowth in \(rms\) Mutants Is Restored in Isolated Nodes**

In this work, we unexpectedly found that buds on isolated node explants of \(SL\)-defective \(rms\) pea mutants have a normal auxin response (Fig. 1), whereas similar experiments on Arabidopsis \(max\) mutants showed auxin insensitivity (Sorefan et al., 2003) and equivalent buds on decapitated whole pea plants are minimally inhibited by exogenous auxin (Beveridge et al., 2000). The key question is why the presence or absence of roots (and the basal part of the stem) has such a great influence on auxin response. To explore underlying regulatory differences, we compared transcript and hormone levels in bud and stem tissues of wild-type and \(rms\) mutant plants following decapitation, with or without simultaneous root removal. Many of the responses in decapitated plants and explants were similar, especially changes in transcript levels, and are therefore unlikely to be explanatory variables; however, others, particularly CK levels, were divergent. Given that sampling was done up to 6 h after treatment, around the time that buds initiate growth (Morris et al., 2005), it appears that rapid molecular reprogramming occurs. These changes are dependent on tissue type and genotype, and are influenced by auxin and by differences between decapitated plants and explant systems (e.g. roots).

**Multivariate Analysis Tools Allow Visualization of Coregulated Genes and Hormones, and Reveal Contrasts between Treatments and Genotypes**

Many individual changes in transcript and hormone levels are reported here, with significance initially assessed by univariate procedures based on ANOVA (Supplemental Tables S2 and S3). To aid visualization and interpretation of the complex relationships, we subjected all data sets to multivariate analysis. Similar approaches were reported in relation to hormonal regulation of dormancy (Chope et al., 2012) and responses to abiotic stresses (Albacete et al., 2010; Pinheiro et al., 2011). Such analyses facilitate detection of groupings of variables and discrimination of differences between genotypes and treatments. We used the shifts between clusters of genotypes and treatments from the transcript and hormone scores plots (Figs. 3B and 7, B and E) to infer the principal effects associated with decapitation, root removal, auxin response, and SL deficiency. The overall trends are depicted in Figures 3C and 7, C and F, as arrows derived from geometric means of means for each of these factors. Directions of the arrows enable deduction of the main transcripts and hormones affected, by comparing with directions on the loadings plots (Figs. 3A and 7, A and D). Some trends matched predictions, such as the opposite responses to auxin and decapitation for auxin transporter and \(IPT\) expression in stems, with the latter positively correlated with levels of stem CKs.

Several broader relationships were revealed, especially clustering of subsets of transcripts or hormones across the whole data set that may relate to coregulation at the biochemical or molecular level. In addition to attempting to discover new regulatory differences between wild-type plants and \(SL\) mutants, we focused especially on possible reasons for the dramatic difference in auxin response in buds of \(rms\) mutants between decapitated plants and explants. The multivariate transcript analysis (Fig. 3) included the bud dormancy markers DORMANCY-ASSOCIATED PROTEIN1 (DRM1) and DRM2 (Supplemental Text S1; Supplemental Fig. S1), revealing trends that are partially consistent with previous reports showing decreased expression in buds coincident with growth initiation (Stafstrom et al., 1998; Balla et al., 2011). However, changes did not always

---

**Figure 6.** (Continued.)
sampled after 6 h except intact plants (time 0). Auxin was supplied as IAA (1500 \(\mu g\) \(g^{-1}\) in lanolin). White bars, The wild type (\(P. sativum\) 'Pavus'); light gray bars, \(rms\)1-1; and dark gray bars, \(rms\)2-2. Values are plotted as the mean ± se. \(n = 3\) biological replicates. IAA, Indole-3-acetic acid; WT, wild type.
Figure 7. Differential regulation of CK levels in stem tissues. Principal components-based factor analysis showing the first three factors that together account for 83% (approximately 49%, 22%, and 13%, respectively) of the total variance. A to C, Factors 1 and 2. D to F, Factors 2 and 3. Factor 1 relates largely to the gross changes caused by decapitation and auxin, whereas factors 2 and 3 distinguish different CK types, and are associated with the effects of root removal. A and D, Loadings plots for each CK.
Genes for Auxin Transport and CK Biosynthesis in Stems Are Regulated in a Largely SL-Independent Manner

Transcript levels for auxin transporters and CK biosynthesis genes showed dynamic changes in stems, and were greatly influenced by decapitation, node isolation, and exogenous auxin but were minimally affected by SL deficiency (Figs. 2, 3, and 5). Shoot tip removal resulted in depletion of PsPIN1 transcripts (Fig. 2A) with broadly similar but smaller reductions in PsPIN2 and PK2 (Supplemental Fig. S1, A and B). These reductions were largely prevented by auxin in all genotypes, yet auxin did not prevent bud outgrowth in decapitated rms plants. A strong correlation among the auxin genes was revealed by the clustering in Figure 3A, implying transcriptional coregulation of several components of the polar auxin transporter system in stems. The relatively small impact of SL mutations on auxin transporter expression in stems contrasts with elevated PIN transcript abundance in Arabidopsis SL mutants (Bennett et al., 2006). However, SL effects on auxin transport may be largely at the protein level through influences on PIN abundance and localization (Bennett et al., 2006; Crawford et al., 2010).

Consistent with previous data on wild-type peas (Tanaka et al., 2006), stem PsIPT1 and PsIPT2 trends were highly correlated with each other (Fig. 3A), but opposite to those for auxin transporter genes, with massive upregulation following decapitation or node isolation and suppression by auxin (Fig. 5, A and B). Notably, these changes were largely unaffected by SL-related mutations. Insensitivity of stem PsIPT expression to SL defects is consistent with the failure of exogenously supplied SL to modify PsIPT expression (Dun et al., 2012).

These findings support a model in which the shoot tip and auxin supply is the major influence on regulation of genes for auxin transport and CK synthesis, with SLs playing a relatively minor role. In relation to second messenger components of shoot branching models, it can be deduced that auxin does not regulate CK biosynthesis through its effects on SL levels. Importantly, because auxin transporter gene and IPT profiles in stems were similar between explants and decapitated plants of rms mutants, we conclude that altered expression of these genes is not the primary cause of the differential growth response to auxin. It remains possible in pea that additional undiscovered members of these gene families or posttranscriptional regulation may have significant roles.

Conditional Auxin Response in rms Mutants May Be Due to Additional Root-Supplied Zeatin CKs

At least three types of difference may explain the suppressed auxin response in decapitated rms mutants that contrasts with normal responses in explants. First, the inhibitory influence of exogenous apical auxin may require an additional factor that acts at the node or bud but is supplied by the roots. SLs are candidates here because their biosynthesis is restored or enhanced by auxin (Foo et al., 2005), decapitated rms1 and rms2 plants show diminished auxin responses, and SLs are translocated from root to shoot (Kohlen et al., 2011). However, the SL biosynthetic gene RMS1 is also expressed locally in stems (Foo et al., 2005), and shows equal transcriptional response to auxin in both decapitated and explant experiments (Fig. 4), suggesting that absence of SL in rms mutants is unlikely to account for the differential bud growth responses to auxin treatment. A second possibility is that auxin transport may differ below the point of decapitation depending on whether the system is a decapitated plant or an explant. For example, depletion of auxin in the stem may be attenuated in explants due to disruption of the auxin transport system at the basal end. However, SL mutants have generally enhanced auxin transport (Beveridge et al., 2000; Bennett et al., 2006; Cazzonelli et al., 2009), and we found no obvious difference in PIN gene expression between explants and decapitated plants. A third option is a root-derived branch-promoting signal, such as CK, that overrides the inhibitory effects of auxin (Chatfield et al., 2000). In SL-deficient rms mutants, alternative auxin targets such as suppression of local CK biosynthesis may provide the explanation for bud inhibition in isolated rms nodes. By contrast, additional CK arriving from roots of decapitated plants (Foo et al., 2007) is significantly redirected to bud tissues by decapitation (Mader et al., 2003) and may not be inhibited by auxin. Supporting evidence comes from the differences in CK profiles: Auxin suppressed tZ-type CKs in decapitated stems much less than in corresponding explants, whereas levels of other CKs were comparable between the two treatments. The tZ-type CKs are highly bioactive and are the predominant

Figure 7. (Continued.)

type. Ellipses in (D) depict groupings according to CK side chain type. B and E, Scores plots for each sample with treatment clusters highlighted by boundary lines, annotated as for Figure 3B. C and F, Overall impacts of treatments and genotype, based on mean groupings on score plots (B and E). Lengths of arrows are derived from differences in mean of means between treatments and indicate relative strength of response to decapitation, auxin and root removal. Directions of arrows align with compounds that show maximal variation, both positive and negative, thus indicating principal variables underlying each treatment comparison. In (C), auxin does not exactly reverse the decapitation response. In (F), auxin response is orthogonal to (independent of) the impact of root removal. 1, rms1; 2, rms2; A, cut + auxin; D, cut; E, isolated nodal explant; I, intact; R, decapitated with roots; W, wild type. [See online article for color version of this figure.]
forms translocated in the xylem (Foo et al., 2007; Hirose et al., 2008). Moreover, exogenous CKs delivered via the xylem stimulate bud outgrowth of SL mutants more than in wild-type plants (Dun et al., 2012). On this basis, we propose that the additional tZ-type CKs arriving from roots in decapitated SL-deficient shoots might be sufficient to promote bud outgrowth. In comparison, explants lack a long-distance CK supply, and rely solely on local CK biosynthesis, with the latter being effectively suppressed by auxin through its SL-independent effects on IPT expression.

Sources and Regulation of Shoot CKs

CKs in bud and stem tissues are likely to derive from a combination of local biosynthesis and import, including delivery from roots via the xylem. In addition, stem CKs could move into buds. Because rms1 and rms2 plants have low and high xylem CKs, respectively (Beveridge et al., 1997b), these mutants would be affected in quantities of root-supplied CKs. In Figure 6, the 2-fold increase in total CK in intact rms1 stems is due entirely to IP compounds, whereas tZ compounds are the main CKs delivered in xylem sap. Conversely, the increased CKs in rms2 stems are primarily tZ types, which are likely delivered from elevated xylem CK of this mutant. The 2-fold increased IPT1 expression in rms1 stems (Fig. 5A) is also seen in other SL mutants (Dun et al., 2012) and is consistent with local compensation for reduced xylem supply. By contrast, rms2 has additional systemic CK supply and no increase in local IPT. In addition, rms1 is hypersensitive to CK (Dun et al., 2012), suggesting that absence of SL results in elevated CK response, which might affect bud outgrowth. Distinctly, between local and imported CKs is further aided by comparing profiles from decapitated plants and explants, because any increase in total CK content of the latter must be due to enhanced local biosynthesis, decreased degradation, or possibly release from conjugated forms. Hydrolysis of CK O-glucosides could theoretically contribute to bioactive pool sizes, but levels of glucosylated CKs were low and do not appear to be a major factor here.

In stems, changes in PsIPT transcripts (Fig. 5) correlated well with trends in CK content (Fig. 6), especially IP-type CKs such as IPRP, the immediate product of IPT enzymes, implicating predominantly local biosynthesis. In particular, CK and IPT levels both increased substantially following decapitation or node isolation, and auxin had strong suppressive effects. These changes appear to be largely SL independent as they were not greatly affected by genotype, similar to responses seen in rice (Zhang et al., 2010). By contrast, IPT expression was greatly reduced in rms buds and did not correlate with bud CK levels (Figs. 5 and 6). At least part of the bud CK pool may therefore be imported from sub-tending stem tissue or roots. Indeed, Dun et al. (2009) used modeling approaches to predict that xylem CKs contribute to promotion of branching especially in SL-deficient backgrounds.

Bud-Specific SL-Dependent Responses and Bud Growth Status

The transcript profiles in buds sharply contrasted with those in stems, with genes such as PsAUX1 and PK2 showing consistently lower expression in rms mutant buds compared with the wild type (Figs. 2 and 4; Supplemental Fig. S1). The dramatic depletion of PsAUX1 transcripts in rms buds even in intact plants and strong correlation with PK2 expression was unexpected, as was the stability of expression of both genes across all treatments including auxin application. Because these treatments differentially affect bud outgrowth, altered expression of auxin-related genes in buds does not appear to be a major factor in growth control here.

The strong influence of SL deficiency on expression of PsIPT genes in buds likewise differs from stem profiles. This suggests that in buds, these genes are largely buffered against influences from the stem, but their normal expression requires SL. The mechanisms of regulation of these tissue-specific differential expression patterns are not yet clear. However, shoot branching may be oppositely regulated by SL and CK via their direct effects on expression of the transcription factor gene PsBRC1 in buds (Braun et al., 2012; Dun et al., 2012). BRC1 in turn may affect local CK levels through functions in regulation of the cell cycle (González-Grandío et al., 2013). In addition, it has long been known that auxin in the polar transport stream in the stem does not enter axillary buds (Everat-Bourbouloux and Bonnemain, 1980), so direct effects of stem auxin depletion on bud IPT expression would not be expected.

CONCLUSION

This work reveals that, independent of SL, auxin reverses down-regulation of auxin transporter expression and up-regulation of CK biosynthesis gene expression after decapitation or node isolation. Because both treatments had similar effects, these gene sets cannot explain the differential bud growth control by auxin in SL-deficient rms mutants. Such findings can allow refinement of current shoot branching models. Specifically, our data indicate that SL is likely not the second messenger in auxin-regulated CK biosynthesis.

Possible explanatory differences between auxin responses in decapitation and node isolation experiments on rms plants were highlighted through multivariate analysis of CK profiles. In particular, auxin had a much stronger suppressive effect on levels of tZ-type CKs in explants than in decapitated plants. The failure of auxin to inhibit branching in decapitated rms mutant plants is proposed to be due to auxin suppressing local CK biosynthesis and accumulation, but being less effective at preventing CKs arriving from the roots or cotyledons. Especially in mutants with SL defects, this source of CKs may act as a positive signal for bud outgrowth in decapitated plants, but is absent in auxin-treated explants. The effectiveness of the imported CKs is seen only in SL-deficient rms mutants, presumably
because auxin-induced up-regulation of SL in wild-type plants provides a dominant inhibitory signal. Further studies on CK gene expression, transport, and metabolism are required to evaluate the regulation and importance of both local and long-distance CK supplies for shoot branching. The sugar status of the shoot is another important variable (Mason et al., 2014) that may differ between decapitated plants and isolated segments, and should be considered in future work such as testing whether sugars regulate CK levels and/or signaling. Additional distinctions are likely to exist between conserved rapid responses to decapitation and the ontogenetic branching patterns that contribute to diverse shoot architectures over much longer time scales.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

The wild-type pea (Pisum sativum L.) was immediately applied to the cut stump. For auxin treatments, 1500 μg g⁻¹ indole-3-acetic acid in lanolin was applied. After 1 or 2 h, the apical ends including all auxin-lanolin mixture were excised and discarded. Pools of 10 (transcript analysis) or 20 (hormone analysis) bud samples were removed from the excised nodes using a microscapel and were weighed and frozen in liquid nitrogen. Ten-mm lengths of the remaining stem tissues, centered on node 3 were cut from 12-d-old plants and their basal ends were inserted into gel medium (Phytagel 6 g/L, one-half strength Murashige and Skoog complete nutrients). Auxin treatments were applied as described except samples were ground under liquid nitrogen using micropestles, and 5 ng of internal standards were added. LC-MS analysis methods were adapted from Foo et al., (2007). Samples were injected onto a Phenomenex 3 μm C18 Luna 100 × 2-mm column on an Agilent 1100 Binary LC system, coupled to an Applied Biosystems Q-Trap hybrid mass spectrometer fitted with a Turbolonspray (electrospray) source operating in positive ion MRM mode. The solvent program was a gradient of acetonitrile in 10 ms ammonium acetate (pH 3.4), initially 5% for 4 min, rising to 14% at 20 min and 32% at 25 min, using a flow rate of 200 μL min⁻¹. Cks were detected through MRM, with a dwell time of 30 ms for each tandem mass transition ion pair. The signal to noise ratio was improved by using the scheduled scan mode in which each MRM signal is monitored only for a 2- or 4-min window centered on the expected retention time of the target compound. The list of compound names, abbreviations, and MRM mass-to-charge values is given in Supplemental Table S1.

**Gene Expression Analysis**

Frozen stem tissue (150 mg) was ground under liquid nitrogen, and RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s guidelines with some modifications. In short, 1 mL of TRIzol was added to powdered tissue and left to stand for 5 min. Samples were centrifuged at 12,000g for 10 min at 4°C, then the supernatant was removed, shaken for 15 s with 200 μL of chloroform, and incubated at room temperature for 2 to 3 min. After centrifugation as above, the aqueous layer was removed and RNA was precipitated by mixing with 250 μL of isopropanol, 250 μL of 0.8 M sodium citrate, and 1.2 M sodium chloride. After 10 min at room temperature, samples were centrifuged again. After discarding the supernatant, the pellet was washed with 1 mL of 75:25 (v/v) ethanol to diethyl pyrocarbonate (DEPC)-treated water. Samples were centrifuged for 5 min at 7,500g and 4°C, and the ethanol was carefully removed. RNA pellets were then air dried and resuspended in 20 μL of DEPC-treated water. RNA isolation from bud samples was essentially the same, using one-half the volume of TRIzol reagent to weight of tissue ground using a micropestle, with resulting RNA pellets being resuspended in 10 μL of DEPC-treated water. Total RNA (1 μg), quantified by a UV spectrophotometer, was treated with 1 unit of DNase (Fermentas) and first-strand complementary DNA (cDNA) was synthesized with SuperScript III reverse transcriptase according to the manufacturers’ guidelines using 500 ng μL⁻¹ of oligo(dT), 300 ng μL⁻¹ of random hexamers, and 40 units of RNAseOUT (Invitrogen).

Gene-specific primers (Supplemental Table S4) were designed manually from database sequences, and the reference gene used was 18S RNA. PCR products were confirmed for all genes via TA cloning (Invitrogen) and sequencing. PCR reactions were carried out with an aliquot of each cDNA sample, using the QuantTect SYBR Green kit (Quagen) in a 7900HT Sequence Detection System (Applied Biosystems). PCR reactions contained 400 μL of each primer in a 20 μL reaction volume under the following conditions: 95°C for 10 min, then 45 cycles of 95°C for 15 s followed by 60°C for 1 min, and the dissociation step, 95°C for 2 min, 60°C for 15 s, and lastly, 95°C for 15 s. Control reactions either omitting the template, or using samples in which reverse transcriptase was omitted from the cDNA synthesis reaction, were performed to confirm the specificity of the primer sets. The average expression level for each cDNA sample was normalized against the expression of the reference gene. In all cases, three independent biological replicate samples were analyzed, with two technical replicates performed for each sample.

**Statistical Analysis**

Gene expression and hormone level data sets were subject to univariate and multivariate statistical analyses. Each gene or hormone was first analyzed by one-way and two-way ANOVA using genotype and treatment as the factors, followed by Tukey’s honestly significant difference post hoc tests.
Multivariate procedures were applied using the StatistiXL version 1.9 add-on package (statistiXL) within Microsoft Excel. Of the several procedures that were initially tested, factor analysis based on the principal components method provided the clearest outputs and visualizations. Analyses were conducted on the entire transcript or CK data sets. Transcript data were first log transformed to standardize variances. Factors were extracted from the R correlation matrix, for all factors with eigenvalues > 1. Outputs are represented as plots of scores (individual samples) and loadings (measured variable) values. To aid visual interpretation, treatments, genotypes, or variables that clearly grouped together were highlighted by manually drawing boundary lines. The main effects of treatment and genotype factors were computed as means of mean values and visualized as arrows representing overall directions and magnitudes of shifts.

**Supplemental Data**

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

**Supplemental Figure S1.** Expression of additional auxin transporter and dormancy marker genes in bud and stem tissues.

**Supplemental Figure S2.** Time course of changes in expression of PsIPT genes in stem tissues following decapitation or node isolation.

**Supplemental Figure S3.** Additional cytokinin profiles from stem tissues of decapitated plants and nodal explants.

**Supplemental Figure S4.** Differential regulation of cytokinin levels in stem and bud tissues.

**Supplemental Table S1.** MRM settings for LC-MS analysis of cytokinins.

**Supplemental Table S2.** Cytokinin content univariate analysis by 2-way ANOVA.

**Supplemental Table S3.** Stem transcript univariate analysis by 2-way ANOVA.

**Supplemental Table S4.** Primer details for quantitative reverse transcription-PCR experiments.

**Supplemental Text S1.** DRM gene expression.

**ACKNOWLEDGMENTS**

We thank Joe McKenna, Nicolas Kral, Nicolas Ortiz-Vaquerizas, and Kenny Yeung for technical assistance, Catherine Rameau for critical reading of the article, and the editors and anonymous reviewers for several helpful suggestions.

Received March 23, 2014; accepted June 3, 2014; published June 5, 2014.

**LITERATURE CITED**


Beveridge CA, Symons GM, Murfet IC, Ross JJ, Rameau C (1997b) The rms1 mutant of pea has elevated indole-3-acetic acid levels and reduced root-sap zeatin riboside content but increased branching controlled by a graft-transmissible signal(s). Plant Physiol 115: 1251–1258


Copyright © 2014 American Society of Plant Biologists. All rights reserved.


Mason MG, Ross JJ, Babst BA, Wienclaw BN, Beveridge CA (2014) Sugar demand, not auxin, is the initial regulator of apical dominance. Proc Natl Acad Sci USA 111: 6092–6097


Morris SE, Cox MCH, Ross JJ, Kriatantini S, Beveridge CA (2005) Auxin dynamics after decapitation are not correlated with the initial growth of axillary buds. Plant Physiol 138: 1665–1672


