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Integration of the SMXL/D53 strigolactone signalling repressors in the model of shoot branching regulation in *Pisum sativum*.

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Summary

DWARF53 (D53) in rice (*Oryza sativa*) and its homologs in Arabidopsis (*Arabidopsis thaliana*), SUPPRESSOR OF MAX2-LIKE 6 (SMXL6), SMXL7 and SMXL8, are well established negative regulators of strigolactone signalling involved in shoot branching. Little is known of pea (*Pisum sativum*) homologs and whether D53 and related SMXLs are specific to strigolactone signalling pathways. Here, we identify two allelic pea mutants, *dormant3 (dor3)*, and demonstrate through gene mapping and sequencing that DOR3 corresponds to a homolog of D53 and SMXL6/SMXL7, designated PsSMXL7. Phenotype analysis, gene expression, protein and hormone quantification assays were performed to determine the role of PsSMXL7 in bud outgrowth regulation and the role of PsSMXL7 and D53 in integrating strigolactone and cytokinin responses. Like D53 and related SMXLs, we show that PsSMXL7 is strigolactone degradable, and induces feedback upregulation of *PsSMXL7* transcript. Here we reveal a system conserved in pea and rice, whereby CK also upregulates *PsSMXL7/D53* transcripts, providing a clear mechanism for strigolactone and cytokinin cross-talk in the regulation of branching. Further deepening our understanding of the branching network in pea, we provide evidence that strigolactone acts via PsSMXL7 to modulate auxin content via *PsAFB5*, which itself regulates expression of strigolactone biosynthesis genes. We therefore show that PsSMXL7 is key to a triple hormone network involving an auxin-SL feedback mechanism and SL-CK crosstalk.

INTRODUCTION

Shoot branching is an important plant developmental process that is highly regulated by both environmental and endogenous signals, including the plant hormone strigolactone (SL). SLs are a group of structurally similar carotenoid-derived compounds that regulate many plant developmental processes including, but not limited to, shoot branching (Brewer *et al.*, 2013). Much has been discovered about SL biosynthesis and signalling through the identification and investigation of branching mutants in numerous species, including the recent identification of SL signalling targets: DWARF53 (D53) in rice (*Oryza sativa*), SUPPRESSOR OF MAX2-LIKE (SMXL) in Arabidopsis (*Arabidopsis thaliana*) and TaD53 in wheat (*Triticum aestivum*) (Jiang *et al.*, 2013; Zhou *et al.*, 2013; Soundappan *et al.*, 2015; Wang *et al.*, 2015; Liu *et al.*, 2017). These

signalling components seem conserved in seed plants (Walker *et al.*, 2019), however, whether they are regulated by other signalling pathways that regulate shoot branching is still to be determined.

SL perception involves an α/β hydrolase (RAMOSUS3 (RMS3) in pea (*Pisum sativum*), AtD14 in Arabidopsis, D14 in rice), which cleaves SLs and covalently traps one of the cleavage products (Hamiaux *et al.*, 2012; Yao *et al.*, 2016; de Saint Germain *et al.*, 2016; Yao *et al.*, 2018). This attachment induces a conformational change to the receptor allowing the recruitment of partners for downstream signalling: an F-box protein (RMS4 in pea, AtMAX2 in Arabidopsis, D3 in rice) which forms part of a Skp, Cullin, F-box (SCF) complex, and D53 in rice (AtSMXL6, AtSMXL7, and AtSMXL8 in Arabidopsis) (Shabek *et al.*, 2018). D53, AtSMXL6, AtSMXL7, and AtSMXL8 proteins are polyubiquitinated by $SCF^{D3/AtMAX2}$ in the presence of SL and degraded by the 26S proteasome (Jiang *et al.*, 2013; Zhou *et al.*, 2013; Soundappan *et al.*, 2015; Wang *et al.*, 2015). *d53, Atsmxl6* and *Atsmxl7* gain-of-function mutants contain a mutation that prevents SL degradation of D53, AtSMXL6 and AtSMXL7 proteins, respectively, resulting in increased branching phenotypes (Jiang *et al.*, 2013; Zhou *et al.*, 2013; Soundappan *et al.*, 2015; Liang *et al.*, 2016). While loss-of-function *Atsmxl6 Atsmxl7 Atsmxl8* double and triple mutants do not alter rosette branching in a wild-type (WT) background, they suppress the increased branching phenotype observed in SL biosynthesis and perception mutants (Soundappan *et al.*, 2015; Wang *et al.*, 2015). Therefore, D53 in rice and AtSMXL6, AtSMXL7 and AtSMXL8 in Arabidopsis act downstream of SL to promote shoot branching.

D53, AtSMXL6, AtSMXL7 and AtSMXL8 also interact with the transcriptional co-repressors, TOPLESS (TPL) and TOPLESS-RELATED (TPR) proteins (Jiang *et al.*, 2013; Zhou *et al.*, 2013; Soundappan *et al.*, 2015; Wang *et al.*, 2015). In conjunction with TPL and/or TPRs, D53 and AtSMXL6, AtSMXL7 and AtSMXL8 probably repress SL transcriptional targets in the absence of SL (Smith and Li, 2014). AtSMXL6, AtSMXL7 and AtSMXL8 also function as transcription factors in Arabidopsis, binding DNA directly and negatively regulating their own transcription (Wang *et al.*, 2020). The well characterised SL-response gene *BRC1* (*BRANCHED1; FINE CULM1* (*FC1*)/*OsTEOSINTE BRANCHED1* (*TB1*) in rice) is a likely transcriptional target of D53 and AtSMXL6, AtSMXL7 and AtSMXL8, because its expression is downregulated in gain-of-function *d53* mutants and upregulated in loss-of-function *Atsmxl6 Atsmxl7 Atsmxl8* triple mutants (Zhou *et al.*, 2013; Soundappan *et al.*, 2015; Wang *et al.*, 2015). Chromatin immunoprecipitation sequencing assays demonstrated that AtSMXL6 was able to bind to the promotor region of *AtBRC1* but very likely not directly (Wang *et al.*, 2020). New insights indicate that the

downstream mechanisms of action for D53/SMXL proteins differ in monocotyledonous and dicotyledonous species (Wang *et al.*, 2020). In rice, IDEAL PLANT ARCHITECTURE1 (IPA1)—otherwise known as SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 14 (OsSPL14) and a member of the SPL family transcription factors—directly binds to the promoter of *OsTB1* to suppress rice tillering (Lu *et al.*, 2013). Moreover, IPA1 enhances *D53* expression as a feedback mechanism (Song *et al.*, 2017). In wheat, the physical interaction of the SL repressor TaD53 with the TaSPL3 and TaSPL17 proteins represses TaSPL3/TaSPL17-mediated transcriptional activation of *TaTB1* expression (Liu *et al.*, 2017). In contrast, the closest homologs of IPA1 in Arabidopsis, AtSPL9 and AtSPL15, likely regulate shoot branching independently from SL (Bennett *et al.*, 2016; Wang *et al.*, 2020).

SL biosynthesis is under negative feedback regulation via a shoot-root signal, demonstrated by SL mutants in many different species exhibiting increased expression of SL biosynthesis genes (Foo *et al.*, 2005; Snowden *et al.*, 2005; Zou *et al.*, 2006; Arite *et al.*, 2007; Liang *et al.*, 2010; Proust *et al.*, 2011; Guan *et al.*, 2012) and by SL treatment reducing expression of these genes (Umehara *et al.*, 2008; Wen *et al.*, 2015; Mashiguchi *et al.*, 2009; Liang *et al.*, 2010; Proust *et al.*, 2011; Guan *et al.*, 2012; Dun *et al.*, 2013). Auxin is the most likely candidate for this shoot-root feedback signal, because SL mutants also have increased indole-3-acetic acid (IAA) content (Beveridge *et al.*, 1996; Beveridge *et al.*, 1997) and auxin treatment upregulates expression of SL biosynthesis genes (Arite *et al.*, 2007; Hayward *et al.*, 2009; Liang *et al.*, 2010). SLs reduce IAA content in stems by repressing expression of IAA biosynthesis genes; this requires RMS3 and RMS4 but is unlikely to involve PsBRC1 (Ligerot *et al.*, 2017), and it is unknown whether D53/SMXLs are involved.

By reducing IAA content in stems, SL likely prevents the feedback upregulation of SL biosynthesis genes. Auxin regulation of SL biosynthesis involves the auxin receptor F-box protein PsAFB4/5 (RMS2) in pea and AFB4/5 in Arabidopsis. *Psafb4/5* and *afb4/5* mutants display increased branching and dwarf phenotypes (Beveridge *et al.*, 1994; Ligerot *et al.*, 2017). These phenotypes may result from reduced SL levels, because expression of the SL biosynthesis genes, *RMS1/PsCCD8* and *RMS5/PsCCD7*, is reduced in *Psafb4/5* stems compared to WT (Foo *et al.*, 2005; Johnson *et al.*, 2006; Ligerot *et al.*, 2017) and because SL treatment to *Psafb4/5* axillary buds is able to repress their outgrowth (Dun *et al.*, 2009).

In addition to SL, environmental signals (e.g. light, nutrients and decapitation of the shoot apex), developmental signals (e.g. flowering and phase transition), and endogenous signals (e.g.

cytokinin-CK, auxin, sucrose, abscisic acid-ABA and gibberellin-GA), all influence shoot branching (Rameau *et al.*, 2015; Barbier *et al.*, 2019). These different signals and their signalling pathways likely interact to determine optimal shoot branching architecture in response to various cues. For example, BRC1 integrates light, decapitation, sucrose, CK and SL signalling to regulate shoot branching (Rameau *et al.*, 2015). The hormones CK and SL antagonistically regulate *BRC1* expression in ways correlated with their opposing effects on shoot branching: SL inhibits shoot branching, while CK promotes shoot branching (Dun *et al.*, 2012). Therefore, BRC1 allows the plant to integrate both signals in its branching response.

Here, we identify *Pssmxl7* loss-of-function mutants in pea (*Pisum sativum*) that suppress branching to almost WT levels in the SL perception mutants, *rms3* and *rms4*. We show that PsSMXL7 is involved in the transcriptional regulation of *PsBRC1, PsSMXL6, PsSMXL7* and *PsSMXL8*, and we confirm that—similar to D53 in rice and AtSMXL6, AtSMXL7 and AtSMXL8 in Arabidopsis—PsSMXL7 is also degraded by SL in a concentration- and time-dependent manner. Interestingly, we find that CK increases *PsSMXL7* transcript levels, demonstrating that *D53/SMXL* transcript regulation is not specific to SL. Furthermore, we implicate PsSMXL7 in the regulation of auxin levels and in the increased branching phenotype observed in *Psafb4/5* mutants.

Results

Identification of *PsSMXL* homologs

To identify genes acting downstream of *RMS3* and *RMS4* in the regulation of shoot branching, we performed an EMS mutagenesis suppressor screen for reduced shoot branching on *rms3-4* and *rms4-3* pea lines. Two lines—*dor3-1* obtained on an *rms3-4* background (Figure 1, Figure S1) and *dor3-2* obtained on an *rms4-3* background (Figures S2, S3)—almost fully restored the increased branching phenotype of *rms3-4* and *rms4-3* to that observed in WT. *dor3-1* restored the reduced plant height phenotype of *rms3-4* and *rms4-3* to WT levels (Figure 1b, Figure S2b), while *dor3-2* had no effect on *rms4-3* plant height (Figure S2b). This rescue of SL mutant branching phenotypes is reminiscent of the *smxl6, smxl7* and *smxl8* double and triple mutants in Arabidopsis, which can rescue the branching phenotypes observed in the SL mutants, *max2* and *max3* (Soundappan *et al.*, 2015; Wang *et al.*, 2015), and provided us with potential gene candidates for *DOR3*.

To determine if the pea DOR3 protein is indeed a homolog of AtSMXL6/7/8 and D53 proteins, we first searched the pea RNA-seq gene atlas

(https://urgi.versailles.inra.fr/download/pea/Pea_PSCAM_transcriptome/; (Alves-Carvalho *et al.*, 2015) for pea proteins with sequence homology to AtSMXL6/7/8 and D53. We later used sequences from the recent pea genome reference (Kreplak *et al.*, 2019). We identified nine putative pea homologs: PsCam004549/Psat6g084880.1, PsCam006628/Psat6g166560.1, PsCam036166/Psat4g013520.1, PsCam037381/Psat6g049000.1, PsCam042379/Psat5g158160.1, PsCam042433/Psat5g129160.1, PsCam042479/Psat1g144800.1, PsCam042519/Psat2g058160.3, and PsCam044945/Psat4g010720.1 (Figure S4). Phylogenetic analysis of D53 and SMXL proteins in rice, Arabidopsis and pea indicated that three pea proteins, Psat5g158160.1, Psat2g058160.3, and Psat5g129160.1, were in the same clade as D53 and AtSMXL6/7/8 (Figure S4). Psat5g129160.1 was most similar to AtSMXL8, sharing 93% protein sequence identity, so we designated Psat5g129160.1 as PsSMXL8. Phylogenetic analysis was unable to resolve the relationship between the pea proteins, Psat5g158160.1 and Psat2g058160.3, and the Arabidopsis proteins, AtSMXL6 and AtSMXL7 (Figure S4). Protein sequence analysis revealed that the highly conserved RGKT motif, found in D53 and AtSMXL6/7/8—thought to be essential for SL-mediated D53/SMXL protein degradation (Jiang *et al.*, 2013; Zhou *et al.*, 2013; Soundappan *et al.*, 2015; Wang *et al.*, 2015)—was fully conserved in PsSMXL8, while only the lysine and threonine residues were conserved in Psat5g158160.1 and Psat2g058160.3 (Figures 2c and S5).

To determine if the *dor3-1* mutation was in a putative *PsSMXL* gene, we first mapped the *dor3-1* mutation on Linkage Group (LG) III (chromosome 5) of the pea map near the RAPD marker T12_1000 using an F2/F3 population of 96 individuals between (*dor3-1 rms3-4* x *rms3-2*) and Bulk Segregant Analysis (Figure S6). PsCam042519 (*PsSMXL6*) was located on LG I of the pea map (Tayeh *et al.*, 2015) and we designed a Cleaved Amplified Polymorphic Marker (CAPS) marker to map PsCam042433/Psat5g129160.1 (*PsSMXL8*) on LG III (chromosome 5) of the pea map—between markers J11_1000 and AA278 (Figure S6)—but the location of PsCam042379/Psat5g158160.1 (*PsSMXL7*) was unknown at this time. Sequencing of Psat5g158160.1 (*PsSMXL7*) and Psat5g129160.1 (*PsSMXL8*) in the two suppressor mutants, *dor3-1* and *dor3-2*, led to the identification of mutations in the same gene, Psat5g158160.1 (*PsSMXL7*). In the *dor3-1* mutant, a G to A transition at position 1482 after the ATG of the mRNA produced a stop codon and truncated 493 amino acid protein (Figure 2a, b). For the *dor3-2* mutation, a C to T transition was identified at position 3112 giving a stop codon 38 amino acids before the stop codon of the WT protein (Figure 2a, b).

PsSMXL7 regulates shoot branching downstream of SL perception and upstream of _PsBRC1_ in pea

Mutant phenotypic analysis demonstrated that the *dor3-1* mutation could almost fully restore total lateral length of axillary branches and total number of branches in both the *rms3-4* and *rms4-3* mutants to WT levels (Figures 1, S2), and that this restoration occurred at most nodes along the main stem (Figures S1, S3). Similar to *smxl6, smxl7* and *smxl8* single loss-of-function mutants in Arabidopsis (Soundappan *et al.*, 2015; Wang *et al.*, 2015), the *dor3-1* single mutant had no effect on total lateral length (Figure 1c) or number of branches (Figure 1d). Since the *dor3* mutations in Psat5g158160.1 could almost fully restore branching in both the *rms3-4* and *rms4-3* mutants, and *SMXL7* has the largest influence on branching out of *SMXL6/7/8* in Arabidopsis (Soundappan *et al.*, 2015), we designated Psat5g158160.1 as *PsSMXL7*, *dor3-1* as *Pssmxl7-1* and *dor3-2* as *Pssmxl7-2*.

PsBRC1 is a transcription factor that acts downstream of SL to regulate shoot branching in pea and other species (Barbier *et al.*, 2019). The role of PsBRC1 in shoot branching is exemplified by the increased branching phenotype displayed by *Psbrc1* mutants in pea (Braun *et al.*, 2012), Arabidopsis (Aguilar-Martínez *et al.*, 2007; Finlayson, 2007), rice (Minakuchi *et al.*, 2010), maize (Kebrom and Brutnell, 2015; Hubbard *et al.*, 2002) and potato (Nicolas *et al.*, 2015). As previously reported, *d14* mutants in rice and Arabidopsis have decreased expression of *BRC1* (*FC1* in rice) due to decreased SL signalling (Zhou *et al.*, 2013; Wang *et al.*, 2015). We also found decreased *PsBRC1* transcript level in *rms3-4* mutant buds (Figure 3a). This reduction in *PsBRC1* transcript level in *rms3-4* is restored to WT levels by the *Pssmxl7-1* mutation (Figure 3a), implying that *PsSMXL7* acts downstream of *RMS3* to regulate *PsBRC1* transcript level. Similarly, *PsSMXL6*, *PsSMXL7* and *PsSMXL8* genes are also downregulated in the *rms3-4* mutant but restored to WT levels in the *rms3-4 Pssmxl7-1* double mutant (Figure 3b-d). The restoration of this gene transcript level in *rms3-4 Pssmxl7-1* plant indicates that *PsSMXL7* may be involved in regulation of *PsSMXL* genes downstream of *RMS3*.

To determine if *PsBRC1* indeed acts downstream of *PsSMXL7*, we created *Pssmxl7-1 Psbrc1-1* double mutants and measured their branching phenotype. *Pssmxl7-1* could not rescue number of branches, total lateral length or bud length at any node in the *Psbrc1* mutant (Figures 1, S1). This demonstrates that *PsBRC1* acts downstream of *PsSMXL7* to regulate branching, as expected by current models of SL signalling in pea. Interestingly, despite maintaining shoot branching, plant

height was restored to WT levels in the *Pssmxl7-1 Psbrc1-1* double mutant (Figure 1b), suggesting that *PsBRC1* may not act downstream of *PsSMXL7* to regulate plant height.

PsSMXL6, *PsSMXL7* and *PsSMXL8* tissue-specific expression

PsSMXL7 was expressed in all tissues examined and was highest in the epicotyl and lowest in the roots (Figure 4). This correlates with *D53* expression in rice which is high in the shoot bases of seedlings and low in the roots (Jiang *et al.*, 2013), and with *AtSMXL7* expression in Arabidopsis which is relatively low in the roots (Stanga *et al.*, 2013). *PsSMXL8* displayed a similar transcript level pattern to *PsSMXL7*, with highest transcript level in the epicotyl and lowest transcript level in the roots (Figure S7). *PsSMXL6*, however, had highest transcript level in the bud and stipule and lowest transcript level in the upper internode and apex (Figure S7).

SL degrades PsSMXL7 protein resulting in feedback upregulation of *PsSMXL7* transcript

In rice and Arabidopsis, SL signalling occurs through the degradation of D53 and AtSMXL6/AtSMXL7/AtSMXL8 proteins, respectively (Jiang *et al.*, 2013; Zhou *et al.*, 2013; Soundappan *et al.*, 2015; Wang *et al.*, 2015).

To further investigate the degradation of PsSMXL7 in response to SL, we generated constructs that use the 35S promoter to transiently express PsSMXL7 protein fused to luciferase in *Nicotiana benthamiana* leaves and investigated the effects of (±)-GR24 on luciferase activity. Compared with mock treatment, the relative luciferase activities were dramatically reduced 30 minutes following (±)-GR24 treatment, suggesting that PsSMXL7 is also degraded by SL (Figure 5a). Mutation and/or deletion of the conserved RGKT motif in D53 and AtSMXL6/AtSMXL7/AtSMXL8 proteins prevents SL-induced degradation (Jiang *et al.*, 2013; Zhou *et al.*, 2013; Soundappan *et al.*, 2015; Wang *et al.*, 2015). Although this motif was not as highly conserved in the PsSMXL6, PsSMXL7 and PsSMXL8 sequences (Figure 2c), deletion of the four amino acids at the RGKT site in PsSMXL7 (namely GRKT) was sufficient to prevent SL-induced degradation of the Pssmxl7^{GRKT} protein (Figure 5b). Therefore, similar to both rice and Arabidopsis, SL very likely degrades the PsSMXL7 protein, and this degradation is reliant on the presence of the RGKT/GRKT motif in PsSMXL7.

D53/AtSMXL expression appears to be under negative feedback regulation, as SL biosynthesis and signalling mutants display reduced *D53* expression (Jiang *et al.*, 2013; Zhou *et al.*, 2013) and SL treatment results in increased *D53/AtSMXL* expression (Jiang *et al.*, 2013; Zhou *et al.*, 2013;

Stanga *et al.*, 2013). This negative feedback regulation also appears to be conserved in pea as the SL signalling mutant *rms3-4* (Figure 3c) and SL deficient mutant *rms1-2* (Figure 5c), have reduced *PsSMXL7* transcript level compared to WT, and SL treatment can upregulate *PsSMXL7* in both stems (Figure 5c) and buds (Figure 5d and 6a). Furthermore, we demonstrate that SL upregulation of *PsSMXL7* likely requires the F-box protein RMS4 (Figure 5d) and can occur independently of protein synthesis (using cycloheximide (CHX) treatment) (Figure 5d). This could indicate that *PsSMXL7* transcript upregulation results directly from PsSMXL7 protein degradation and does not involve a *de novo* produced transcription factor.

We also examined SL regulation of *PsSMXL6* and *PsSMXL8* transcript level. While SL upregulated both *PsSMXL6* and *PsSMXL8* transcript levels in buds (Figure S8b,e), only *PsSMXL8* was significantly upregulated by SL in stems (Figure S8a,d). As for *PsSMXL7*, both *PsSMXL6* and *PsSMXL8* upregulation by SL requires RMS4 (Figure S8b,e) and occurs independently of *de novo* protein synthesis (Figure S8b,e) indicating that it may result from PsSMXL protein degradation. Interestingly, in Figure 3b,d we found that *Pssmxl7-1* was able to restore *PsSMXL6* and *PsSMXL8* expression in the *rms3-4* mutant, perhaps suggesting that SL regulation of *PsSMXL6* and *PsSMXL8* expression occurs predominantly through degradation of PsSMXL7 protein.

Cytokinin also regulates *PsSMXL7* transcript levels

Previously it was proposed that the SL and CK signalling pathways converge on the transcription factor PsBRC1 in pea buds to regulate branching, because SL increases *PsBRC1* expression and inhibits branching, while CK decreases *PsBRC1* expression and promotes branching (Dun *et al.*, 2012; Kerr *et al.*, 2020). We examined whether CK regulation of *PsBRC1* may instead occur upstream through CK upregulation of *PsSMXL7* transcription. Indeed, we found that *PsSMXL7* transcript level was increased after BA treatment (Figure 6a,c). Additionally, CK and SL regulation of *PsSMXL7* transcript level appears to be additive (Figure 6a). To rule out the possibility of CK upregulating *PsSMXL7* transcription by repressing *PsBRC1*, we examined *PsSMXL7* transcript level after BA treatment in *Psbrc1-1* mutants. *PsSMXL7* upregulation by CK was reduced in *Psbrc1-1* mutants (Figure 6c), suggesting that CK regulation of *PsSMXL7* transcription is affected by PsBRC1.

We previously demonstrated that CK regulation of *PsBRC1* expression was not essential for CK regulation of sustained bud outgrowth (Braun *et al.*, 2012). To determine if CK regulation of

PsSMXL7 transcript levels is necessary for CK regulation of sustained bud outgrowth, we treated dormant *Pssmxl7-1* node 3 buds with BA and measured the length of the bud 7 days later. BA treatment increased node 3 bud length in both WT and *Pssmxl7-1* mutants (Figure 6b). These results indicate that, similar to *PsBRC1*, CK regulation of *PsSMXL7* is not essential for CK regulation of sustained bud outgrowth.

To test whether CK downregulation of *PsBRC1* transcript level occurs through CK upregulation of *PsSMXL7* transcript, we examined *PsBRC1* expression after CK treatment in *Pssmxl7-1* mutants. We found that CK could downregulate *PsBRC1* expression in *Pssmxl7-1* mutants to the same extent as in WT (Figure 6d). This suggests that CK could regulate *PsBRC1* expression independently of its regulation of *PsSMXL7*. It is also possible that the observed down-regulation of *PsBRC1* expression in *Pssmxl7-1* is mediated by *PsSMXL8* which is also up-regulated by CK (see below; Figure S8f).

To determine whether this CK regulation of *SMXLs* is conserved across species, we examined CK regulation of *D53* in rice. Similarly, CK was able to upregulate transcript level of *D53* in rice shoot bases—although this upregulation was abolished after 2 hours (Figure 7a,b), possibly due to increased negative feedback regulation of D53 protein on *D53* transcription. Interestingly, this negative feedback regulation was not observed in rice calli, where *D53* transcript levels were increased significantly 2 hours after CK treatment (Figure 7b), nor in pea buds where *PsSMXL7* transcript level is upregulated by CK up to 6 hours after treatment (Figure 6c).

We also observed CK regulation of *PSMXL6* and *PsSMXL8* transcription. Unlike SL which upregulated all three *PsSMXL6*, *PsSMXL7* and *PsSMXL8* transcripts, CK had different effects on *PsSMXL6*, *PsSMXL7* and *PsSMXL8* expression. Like *PsSMXL7*, *PsSMXL8* was also upregulated by CK additively to SL (Figure S8f). However, *PsSMXL6* was downregulated by CK antagonistically to SL (Figure S8c), suggesting a different mechanism of CK-regulation than *PsSMXL7* and *PsSMXL8*.

PsSMXL7 is involved in SL regulation of IAA levels and PsAFB4/5-dependent shoot branching

Generally, SL mutants have high IAA levels in shoots compared with WT (Beveridge *et al.*, 1996; Beveridge *et al.*, 1997) and it was recently demonstrated that SLs repress IAA levels in pea stem by down-regulating transcript levels of IAA biosynthesis genes (Ligerot *et al.*, 2017). To

investigate whether the *Pssmxl7-1* mutation can restore IAA to WT levels in SL mutants, IAA was quantified in upper internodes of pea plants. IAA levels were higher in *rms3-4* plants compared to WT, as previously reported, but were reduced to WT levels in *rms3-4 Pssmxl7-1* plants supporting the hypothesis that PsSMXL7 degradation could mediate the down-regulation of IAA levels by SLs (Figure 8).

While SLs repress IAA levels in the shoot, IAA enhances transcript levels of SL biosynthesis genes (Foo *et al.*, 2005; Zou *et al.*, 2006; Johnson *et al.*, 2006; Arite *et al.*, 2007; Hayward *et al.*, 2009; Liang *et al.*, 2010). In pea, it was proposed that the *PsAFB4/5* gene was involved in a shoot-to-root feedback signal that up-regulates transcript levels of SL biosynthesis genes (Beveridge *et al.*, 2009). The *Psafb4/5-1* pea mutant displays increased shoot branching and semi-dwarf phenotypes similar to the *rms* SL-related mutants (Beveridge *et al.*, 1996), and indeed bud outgrowth can be inhibited by treatment of (±)-GR24 to *Psafb4/5* buds (Dun et al., 2009; Brewer *et al.*, 2009). In contrast to SL biosynthesis and response mutants—where *RMS1* transcript levels in the epicotyl are strongly upregulated in comparison to WT—these transcript levels are slightly reduced in *Psafb4/5-1* epicotyl suggesting possible reduced SL levels in this mutant (Foo *et al.*, 2005). To investigate whether *Pssmxl7-1* can reverse *Psafb4/5-1* phenotypes, we compared the phenotypes of *Psafb4/5-1* to those of *Psafb4/5 Pssmxl7-1*. *Pssmxl7-1* could almost fully restore both shoot branching and plant height (Figure 9), indicating that these phenotypes in *Psafb4/5-1* occur predominantly due to reduced SL levels and signalling.

DISCUSSION

Due to its long internodes and easily accessibly buds, pea is an important model system to investigate the regulation of hormone responses for shoot branching. Here, we place SMXL7, the SL target, into the shoot branching model in pea and show that it is critical for integration of SL and CK signalling as well as regulation of auxin level.

Identification of *PsSMXL6, PsSMXL7* and *PsSMXL8* in pea

Two *dor3* mutant alleles were identified from a phenotypic screen for reduction of branching in mutagenized *rms3* and *rms4* populations of 3800 and 4200 M2 families, respectively (Rameau *et al.*, 2002). These two *dor3* mutations were found in the same *PsSMXL* gene (Psat5g158160.1) and both caused significantly reduced branching in SL mutants to almost WT levels. This suggests that *DOR3* may be the main *PsSMXL* acting downstream of SL signalling to control shoot branching,

similar to *AtSMXL7* which is the most important for branching regulation in Arabidopsis (Soundappan *et al.*, 2015; Wang *et al.*, 2015). Therefore, we designated *DOR3* and Psat5g158160.1 as *PsSMXL7*, and Psat2g058160.3 as *PsSMXL6*. Future identification of *Pssmxl6* and *Pssmxl8* mutants will allow us to confirm their relative influence on shoot branching. The position of the *dor3-2* mutation in the C-terminal end of the protein may affect the formation of a complex among SMXL proteins (Khosla *et al.*, 2020) and the relative strong phenotype of the mutant indicates that this complex plays a role in SMXL regulation of shoot branching.

PsSMXL7 acts downstream of *RMS3* and *RMS4* and upstream of *PsBRC1* in the regulation of shoot branching by SL

SL perception in plants requires both an α/β hydrolase receptor RMS3/D14 and an F-box protein RMS4/D3/MAX2 for ubiquitination of target D53/SMXL proteins (de Saint Germain *et al.*, 2013). Mutations in these SL perception genes cause an increased branching phenotype that cannot be inhibited by SL treatment (Gomez-Roldan *et al.*, 2008; de Saint Germain *et al.*, 2016; Dun *et al.*, 2009). In Arabidopsis, mutations in *AtSMXL6, AtSMXL7* and *AtSMXL8* completely restore branching to WT levels in the *max2* mutant (Soundappan *et al.*, 2015). Here, we demonstrated that *Pssmxl7* mutants in pea can inhibit branching in both *rms3* and *rms4* mutants to almost WT level. This indicates that PsSMXL7 acts downstream of both RMS3 and RMS4 to positively regulate shoot branching in pea.

Two main pathways have been identified through which SL is thought to regulate shoot branching. One pathway involves SL upregulation of the *BRC1* transcription factor, while the other pathway involves SL inhibition of PIN1 localization to the plasma membrane and/or SL effect on auxin feedback on PIN1 internalization (Zhang *et al.*, 2020) which prevents auxin transport out of buds (Waldie *et al.*, 2014). In both rice and Arabidopsis, D53 and AtSMXL6, AtSMXL7 and AtSMXL8 repress *FC1/BRC1* transcription (Zhou *et al.*, 2013; Soundappan *et al.*, 2015; Wang *et al.*, 2015; Wang *et al.*, 2020), while AtSMXL6, AtSMXL7 and AtSMXL8 in Arabidopsis promote PIN1 localization to the plasma membrane and increase auxin transport out of buds (Soundappan *et al.*, 2015). We show here that PsSMXL7 represses *PsBRC1* downstream of RMS3. Additionally, the *Pssmxl7* mutation could not restore branching in the *Psbrc1* mutant, indicating that PsBRC1 acts downstream of PsSMXL7 to regulate shoot branching. In fact, branching in the *Psbrc1-1* mutant was completely unaffected by the *Pssmxl7-1* mutation, implying that SMXL7 regulation of branching in pea may occur exclusively through *PsBRC1*. These results contrast with

the dual action of SL and AtSMXL6, AtSMXL7 and AtSMXL8 in shoot branching in Arabidopsis (through regulation of both *BRC1* expression and PIN1 localization), but support other evidence that suggests SL regulation of auxin transport is not as important in the regulation of shoot branching in pea as it is in Arabidopsis (Brewer *et al.*, 2009; Brewer *et al.*, 2015). Nevertheless, the branching phenotype of *Psbrc1* is less severe than the branching phenotype of SL deficient and SL response mutants, indicating that SL may still regulate shoot branching via PsBRC1-independent pathway(s) in pea. Future studies with *Pssmxl6* and *Pssmxl8* may help resolve these PsBRC1-independent pathway(s).

PsSMXL7 is involved in SL repression of IAA levels

In pea, an PsAFB4/5-dependent shoot-to-root feedback signal was proposed that up-regulates transcript abundance of SL biosynthesis genes (Beveridge, 2000), and is negatively regulated by SL perception (Beveridge *et al.*, 2009). Recently, this RMS-dependent signal was proposed to be IAA (Ligerot *et al.*, 2017). Here the restoration of IAA levels in *rms3-4* by *Pssmxl7-1* supports the hypothesis that SLs repress IAA levels via PsSMXL7.

The high shoot branching of the *Psafb4/5-1* mutant may be explained by its altered regulation of transcript levels of SL biosynthesis genes and its high CK content in the xylem sap from the roots. In contrast to SL mutants—where high *RMS1* transcript levels in the epicotyl are found—the *Psafb4/5-1* mutant has low expression of *RMS1*. Rather than this leading to SL deficiency, SL quantification, performed in *Psafb4/5-1* roots and root exudates, showed no SL depletion (Foo *et al.*, 2013). Detailed analysis of *RMS1* transcript levels showed that *Psafb4/5-1* displays reduced levels in comparison to WT mainly at upper nodes of the plant whereas levels are similar in roots and at the base of the plant (Dun *et al.*, 2009). To resolve this, we show here that the *Pssmxl7-1* mutation almost fully restores shoot branching and height in *Psafb4/5-1*, indicating that the *Psafb4/5-1* phenotypes are SMXL7 dependent. This is consistent with the ability of (±)-GR24 to inhibit bud outgrowth in *Psafb4/5-1* mutant buds (Dun *et al.*, 2009). The partial restoration of dwarfism in *Psafb4/5-1* by *Pssmxl7-1* is intriguing, as dwarfism of *Psafb4/5-1* could not be restored by grafting to WT rootstock or by (±)-GR24 feeding hydroponically. *Psafb4/5-1* is a mutant in a member of the auxin receptor family and this could explain the absence of restoration in height as auxin response mutants often display dwarf phenotypes (e.g. *axr1* mutants; (Lincoln *et al.*, 1990)).

***PsSMXL7* is transcriptionally upregulated by SL and CK**

SL upregulates expression of *D53* in rice (Jiang *et al.*, 2013; Zhou *et al.*, 2013) and *AtSMXL6*, *AtSMXL7* and *AtSMXL8* in Arabidopsis (Soundappan *et al.*, 2015; Wang *et al.*, 2015). It was recently shown that the AtSMXL6 protein can bind to the promoters of *AtSMXL6*, *AtSMXL7* and *AtSMXL8* and repress their transcription (Wang *et al.*, 2020), which is released upon SL-mediated degradation of D53/SMXL proteins (Jiang *et al.*, 2013). We have shown here that *PsSMXL6*, *PsSMXL7* and *PsSMXL8* are also upregulated by SL in pea, confirming that SL regulation of these *D53*/*SMXL* genes is conserved in both monocots and dicots. We also demonstrated that CK upregulates D53 and PsSMXL7 transcript levels highlighting the conserved nature of this regulation. Dun *et al.* (2012) demonstrated that CK and SL antagonistically regulate branching in pea, most likely through regulation of the *PsBRC1* transcript. However, we have shown here that CK and SL both upregulate *PsSMXL7* and *PsSMXL8* expression, which seems in conflict with an antagonistic relationship between the two hormones. CK and SL probably act independently in this process because combined SL and CK treatments have an additive effect on *PsSMXL7* and *PsSMXL8* expression. That SL and CK have similar effects on *PsSMXL7* and *PsSMXL8* expression but antagonistic effects on shoot branching may be explained by differences in PsSMXL7 and PsSMXL8 protein levels. SL upregulation of *PsSMXL7* and *PsSMXL8* expression is likely due to negative feedback control as found in rice and Arabidopsis (Jiang *et al.*, 2013; Zhou *et al.*, 2013; Soundappan *et al.*, 2015; Wang *et al.*, 2015; Wang *et al.*, 2020), such that SL degradation of PsSMXL7 and PsSMXL8 results in upregulation of their own expression. Whereas CK regulation of PsSMXL7 and PsSMXL8 protein may occur through upregulation of *PsSMXL7* and *PsSMXL8* expression. This hypothesis would explain how both SL and CK have the same effect on *PsSMXL7* and *PsSMXL8* expression, while having differing effects on downstream expression of *PsBRC1* and shoot branching.

Our results show that the *Pssmxl7-1* mutant still displays an increased branching response to CK treatment, although this response is reduced compared to the WT suggesting that PsSMXL7 may be at least partially required for shoot branching responses to CK. We propose a model in pea in which SL and CK converge on PsSMXL7/8 (Figure 10). In order to achieve an antagonistic response to CK and SL, CK upregulation of *PsSMXL7/8* transcript likely results in an increase in PsSMXL7/8 protein while SL upregulation of *PsSMXL7/8* transcript is a result of SL-mediated degradation of PsSMXL7/8 proteins. PsSMXL7/8 then stimulates shoot branching by inhibition of

PsBRC1 expression. CK most likely also acts through PsSMXL7/8-independent pathways to regulate shoot branching, possibly directly through PsBRC1. This model accounts for the antagonistic effects of combinations of SL and CK treatments on *PsBRC1* expression and bud outgrowth as described in Dun *et al.*, 2012, and explains why the *Psbrc1* mutant can still respond to CK treatment (Braun *et al.*, 2012). Another property of the branching network in pea, which is also observed in several other systems, is the enhanced level of auxin in SL mutant plants (Arite *et al.*, 2007; Agusti *et al.*, 2011). The basis of this in pea is now revealed to also involve PsSMXL7. PsSMXL7 is involved in feedback regulation of SL biosynthesis genes via enhancement of stem auxin IAA levels and via PsAFB4/5.

Experimental procedures

Sequencing and phylogenetic analysis of PsSMXLs

Pea SMXL homologs were identified by BLASTP against the pea RNA-seq gene atlas (https://urgi.versailles.inra.fr/download/pea/Pea_PSCAM_transcriptome/); (Alves-Carvalho *et al.*, 2015) using the AtSMXL6 protein sequence from Arabidopsis. The corresponding sequences were later identified from the pea genome reference (https://urgi.versailles.inra.fr/Species/Pisum; (Kreplak *et al.*, 2019). The primers used for sequencing the pea homologs of the D53 clade are given in Table S1.

Multiple sequence alignment was performed on D53, D53-like and SMXL family proteins in rice, Arabidopsis and pea using ClustalW (Thompson *et al.*, 1994). The phylogenetic tree was assembled in MEGAX (Kumar *et al.*, 2018) using the Maximum Likelihood method based on the Dayhoff matrix based model and the optimal tree was generated.

Plant material, growth conditions and measurements

The plants used in this study were derived from different *Pisum sativum* cultivars. The *rms1-2* (rms1-2T), *rms4-1* (K164) and *rms5-3* (BL298) mutants were previously obtained in cv. Torsdag (Dun *et al.*, 2012). The *rms3-4* (T2-30), *rms4-3* (M3T-946), *Psbrc1* and *Psafb4/5-1* mutants were previously obtained in cv. Térèse (Rameau *et al.*, 1997; Braun *et al.*, 2012; Ligerot *et al.*, 2017). The *dor3-1* mutant was screened in an *rms3-4* (T2-30) mutagenized population and backcrossed six times to *rms3-4* before getting the single *dor3-1* line and other double mutants with *Psbrc1-1* and *Psafb4/5-1*. The *dor3-2* mutant was screened in an *rms4-3* (M3T-946) mutagenized population and backcrossed twice. The different single and double mutants were selected in progenies using the following molecular markers. A derived-Cleaved Amplified Polymorphic Sequence (dCAPS) marker was developed for the *dor3-1* mutation using the primers 5'-CGATGAAGATGTTGACAGATATCTCT-3' and 5'-AGCACTTCCAAAAAGCCAAA and then cleaved with the restriction enzyme *Eam*1104I/*Ear*I. The fragment is cleaved in the *dor3-1* mutant but not in the WT. A CAPS marker was designed for the *rms4-3* mutation using the primers 5'-CACGCTCCGTGGGAACG-3' and 5'-CAAGGCGGCGAAGTCGG-3'. The 316 bp band is cleaved by the restriction enzyme *Tru*I/*Mse*I in the *rms4-3* mutant but not in the WT. The *rms3-4* mutation in the T2-30 line was identified using a CAPS marker with the primers 5'-

CGCTATTTTCCATTGGTTCATAT-3' and 5'-GGAGGCACCGATAAGGATG-3'. The 600 bp fragment is cleaved in the WT with the *Bsh*NI restriction enzyme. The *Psbrc1-1* and *Psafb4/5-1* mutations were identified using CAPS markers described in (Braun *et al.*, 2012) and (Ligerot *et al.*, 2017).

For glasshouse experiments in Figures 5d, 6a,c,d, and S8b,c,e,f seedlings were grown as described in (Ferguson and Beveridge, 2009) in either UQ23 potting mix at 4 per 2L pot (Figures 6a,c,d, S8c,f), or in Green Fingers B2 potting mix at 2 per 2L pot (Figures 5d, S8b,e). For glasshouse experiments in all other figures, seeds were grown in 2 L pots filled with clay pellets, 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-h photoperiod (the natural day length was extended or supplemented during the day when necessary using sodium lamps). For hydroponic treatments in Figures 5 and S8, plants were grown and treated as described in (Boyer *et al.*, 2012; Ligerot *et al.*, 2017).

For treatments to the bud in Figure 6b, 10 µL was applied to the bud containing 50% EtOH, 1% polyethylene glycol (PEG)1450 and 0.5% dimethyl sulfoxide (Sigma) with or without 50 µM 6-benzylaminopurine (BA). For all other treatments to the bud, 10 µL was applied to the bud containing 0.01% Tween-20 and 1% PEG1450 in 6.25% EtOH with acetone used as a solvent for (±)-GR24 and DMSO used as a solvent for BA.

For pea bud measurements, nodes were numbered acropetally from the first scale leaf as node 1. Pea bud length was measured with digital calipers and plant height was measured with a ruler.

Mapping of the *dor3-1* mutation and the *PsSMXL8* gene

The *dor3-1* mutation was mapped using an F2 population of 96 individuals between (*dor3-1 rms3-4* x *rms3-2*). The *rms3-4* mutation has been identified in cv. Térèse (T2-30) and the rms3-2 in cv. Torsdag (K564). Because all F2/F3 plants were fixed for *rms3-4*, the *dor3-1* reduced branching phenotype was easy to identify. Polymorphic markers previously identified between Térèse and Torsdag lines (Laucou *et al.*, 1998) were screened for polymorphisms between two pools of ten homozygous *dor3-1* F2 plants and homozygous F2 WT plants using Bulk Segregant Analysis.

PsSMXL8 was mapped on the 164 recombinant inbred lines of the RIL2 population (Térèse x Champagne) (Bordat *et al.*, 2011). A SNP (G/A located at position 1952 of the coding sequence from the ATG) between Térèse and Champagne was used for mapping and the design of a CAPS marker (primers 5'-TCCAGAAGTCCTTTTCCCGC-3' and 5'-TCGCGTCCATGCGAATCTAT-3'; restriction enzyme *Bsu*15I/*Cla*I).

Auxin measurements

IAA extraction and quantification was performed as described in (Ligerot *et al.*, 2017).

Gene expression analyses

For pea, total RNA was isolated as described previously (Braun *et al.*, 2012; Ligerot *et al.*, 2017; François F. Barbier *et al.*, 2019). RNA was quantified using NanoVue Plus and migrated on gels to determine RNA degradation. cDNA was synthesised using the iScriptTM reverse transcription supermix (BioRad) as per the manufacturer's instructions. qRT-PCR analyses were performed and analysed as previously described (Mason *et al.*, 2014; Ligerot *et al.*, 2017).

For rice, WT (HuiDao5) callus tissue grown for three weeks on solid media at 28°C or shoot base tissue grown on hydroponic culture media for two weeks was used. Total RNA was extracted using the RNAprep Pure Plant kit (Tiangen) and cDNA was synthesised using HiSCript II Q Select RT Supermix for qPCR (Vazyme) as per the manufacturer's instructions. qRT-PCR reactions were performed using ChamQ qPCR reagent kit (Vazyme) and the ABI Prism 7500 Sequence detection system as per the manufacturer's instructions.All primer sequences for qRT-PCR are shown in Table S1. Primer sequences for *PsSMXL6*, *PsSMXL7* and *PsSMXL8* were designed using Primer3 software (Untergasser *et al.*, 2012).

Transient luciferase expression assay

To check PsSMXL7 protein stability, we developed an *in vivo* luciferase detection assay using tobacco (*Nicotiana benthamiana*) plants. PsSMXL7cds was PCR amplified from Pea Térèse background cDNA and the following primers PsSMXL7_attB1 5'-gggacaagtttgtacaaaaaagcaggcttcATGCCGACGCCGGTAAGCACAGC-3' and PsSMXL7_attB2_DS 5' -ggggaccactttgtacaagaaagctgggtcGTTCAAGCTAATTCTAGGTGGAA-3', and then recombined into pDONR221 (Invitrogen). Pssmxl7 ^{GRKT} (deletion of the GRKT motif, amino acid G767 to V772) sequence was obtained by Site-directed mutagenesis experiments using QuickChange II XL Site Directed Mutagenesis kit (Stratagene) and the primer PsSMXL7delGRKTVV 5'-TGCCACAACATGCTTGATTATATTGCTGGCGAG-3'. Then, the coding sequences of *PsSMXL7* and *Pssmxl7^{GRKT}* were amplified using SMXL7-LUC-F-5'-GGGCGGAAAGGAATTCATGCCGACGCCGGTAAG-3' and SMXL7-LUC-R-5'-TAGATCCGGTGGATCCGTTCAAGCTAATTCTAGGTGGAAGGCA-3' primer pairs and

cloned at the C-terminal region of the Firefly luciferase gene at the *EcoR*I and *BamH*I restriction sites into the pCambia1200 35S-LUC vector using In-fusion® cloning system (Clontech) as per the manufacturer's instructions. The luciferase reporter was constructed by modifying pCambia1200 vector by introducing Firefly luciferase and Renilla luciferase coding sequences in the same backbone driven by independent CaMV 35S promoter in the same direction (Sun *et al.*, 2021). The vector simultaneously expresses the Luciferase (LUC) as well as Renilla luciferase (RLUC) gene under the control of the CaMV35S promoter, which was used as an internal transformation control. The plasmids were transformed into the EHA105 *Agrobacterium* strain using electroporation. Three-week-old tobacco plants were transfected with *Agrobacterium* culture harbouring PsSMXL7-LUC or Pssmxl7 ^{GRKT}-LUC constructs as described in (Chen *et al.*, 2008). The leaves were transfected with the *Agrobacterium* strain harboring the SMXL7 construct and kept for three days to achieve transient expression. The hormonal treatments were performed in the transfected leaf tissues at different time points. The leaves were harvested in liquid nitrogen and activities of Firefly luciferase and Renilla luciferase were determined using the Dual-luciferase® reporter assay system (Promega). The LUC activity was calculated by normalizing Firefly luciferase values with Renilla luciferase and presented as relative luciferase values.

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under the following accession numbers: PsSMXL6 (MH507404), PsSMXL7 (MH507405), PsSMXL8 (MH507406).

Data availability statement

Data supporting the findings of this work are available within the article and its supporting information files.

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Author contributions

CR and CAB supervised the experiments. SCK, SP, CAB, CR designed the experiments. SCK, SP, A de SG, J-PP, JS, YL, GA, SC, YB, EAD performed the experiments. SCK, CR analysed the experiments and wrote the manuscript. CR, CAB, and EAD supervised and complemented the writing.

Conflict of interest

The authors have no conflict of interest to declare.

Supporting Information

Figure S1. Branching phenotype of the *dor3-1* mutant.

Figure S2. Branching phenotype of the *dor3-2* mutant.

Figure S3. Branching phenotype of the *dor3-2* mutant.

Figure S4. Phylogenetic tree of the SMXL family from Arabidopsis and pea including D53 (OsD53), D53-like (OsD53-LIKE) from rice and AtHSP101 proteins.

Figure S5. Sequence alignment of Arabidopsis SMXL6, SMXL7 and SMXL8 proteins with the pea homologs.

Figure S6. *PsSMXL7/DOR3* and *PsSMXL8* map at different positions on the pea linkage group (LG) III.

Figure S7. *PsSMXL6* and *PsSMXL8* expression in different tissues.

Figure S8. SL and CK regulation of *PsSMXL6* and *PsSMXL8* expression.

Table S1. List of primers used for qRT-PCR analyses.

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FIGURE LEGENDS

Figure 1. Branching phenotype of the *Pssmxl7-1* (=*dor3-1*) mutant. (**a)** Phenotype and quantification of (**b)** plant height (mm), (**c)** total lateral length (mm), and (**d)** number of branches >5mm of 28-day-old *Pisum sativum* seedlings on a Térèse background. Bar, 85 cm. Data are means ± SE with individual data points plotted (n=11-12). Statistical differences were determined using a one-way ANOVA with a Tukey multiple comparison of means post-hoc test, statistical differences of P< Statistical differences were determined using a one-way ANOVA with a Tukey multiple comparison of means post-hoc test, statistical differences of P<0.05 are represented by different letters. are represented by different letters.

Figure 2. PsSMXL7 genomic and protein structures. (a) Structure of the *PsSMXL7* gene showing location of the two *dor3* mutations and the qRT-PCR primers (black arrows). Bases are numbered from the start codon and are based on the genomic sequence; intron (thin line). (**b)** Structure of the PsSMXL7 protein showing the location of the two *dor3* mutations and the four protein domains and key amino acid motifs within the protein, including the Walker A, Walker B and EAR motifs. (**c)** Conservation of the RGKT motif at the GYVG loop site in Arabidopsis, rice

and pea D53/SMXL protein sequences, adapted from Soundappan *et al* (2015). The S_{YDVII} amino acids are an insertion in the PsSMXL6 sequence after the conserved phenylalanine (F) amino acid.

Figure 3. PsSMXL7 acts downstream of RMS3 to regulate expression of *PsBRC1*, *PsSMXL6*, *PsSMXL7* and *PsSMXL8*. Gene expression for (**a)** *PsBRC1*, (**b)** *PsSMXL6*, (**c)** *PsSMXL7*, and (**d)** *PsSMXL8* in node 2 buds of 7-day-old *Pisum sativum* seedlings on a Térèse background. Expression is represented relative to the WT and was normalised against an *Actin* reference gene. Data are means ± SE with individual data points plotted (n = 4 pools of 20 plants). Statistically significantly different means were calculated using Student t-tests (* denotes P<0.05).

Figure 4. *PsSMXL7* expression in different tissues. *PsSMXL7* expression in roots, epicotyl, basal and upper internodes, nodes, buds, stipules and shoot apex of 20-day-old *Pisum sativum* Térèse WT plants. Expression is relative to an *Actin* reference gene. Data are means ± SE with individual data points plotted (n = 3 pools of 8-12 plants).

Figure 5. SL degrades PsSMXL7 protein and upregulates *PsSMXL7* transcript levels. (a, b) Leaves on 3-week-old tobacco (*Nicotiana benthamiana*) plants were transfected with an *Agrobacterium* culture harbouring **(a, b)** 35S:SMXL7-LUC and/or **(b)** 35S:smxl7-LUC constructs, then treated 3 days after agroinfiltrations with 0 µM, 5 µM or 10 µM (±)-GR24 (SL) and harvested **(a)** 30 minutes or **(a, b)** 120 minutes later. The LUC activity was calculated by normalizing Firefly luciferase values with Renilla luciferase and presented as relative Luc/RLuc values. Data are means ± SE with individual data points plotted (*n* = **(a)** 3 or **(b)** 5) **(a).** Statistical differences were determined at each time point separately using a one-way ANOVA with a Tukey multiple comparison of means post-hoc test, statistical differences of $P<0.05$ are represented by different letters. **(b)** Statistically significantly different means from the mock for each genotype were determined using Student t-tests (* denotes $P<0.05$; ns denotes not significant). **(c)** 3-week-old *Pisum sativum* Torsdag (WT) and *rms1-2* seedlings were treated hydroponically with 3 µM (±)-3'-Me-GR24 (SL) and internodes beneath the shoot apex were harvested after 0, 05, 1, 2, 3, 4 or 6 hours. Expression is represented relative to the WT and was normalised against the *Actin* reference gene. Data are means ± SE with individual data points plotted (*n* = 3 pools of 8-10 plants). Statistical differences were determined using a one-way ANOVA with a Tukey multiple comparison of means post-hoc test, statistical differences of $P<0.05$ are represented by different

letters. **(d)** Node 3 buds of 7-day-old *Pisum sativum rms1-2* or *rms4-1* seedlings on a Torsdag background were treated for 6 hrs with 1 μM (±)-GR24 (SL) and/or 10 μM cycloheximide (CHX). Expression is represented relative to the *rms1* mock and was normalized against the geomean of three internal reference genes: *EF1α*, *GADPH* and *TUB2*. Data are means ± SE with individual data points plotted (*n* = 3 pools of approx. 10-30 plants). Statistically significantly different means from the mock or CHX control were determined using a Kruskal-Wallis rank sum test (*** denotes $P < 0.001$).

Figure 6. CK increases *PsSMXL7* transcript levels, but this is not essential for CK regulation of shoot branching. (a) Node 2 buds of 7-day-old *Pisum sativum rms1-2* seedlings on a Torsdag background were treated for 2 hrs with 50 μM BA (CK) with or without 1 μM (+)-GR24 (SL). Expression is represented relative to the mock and was normalized against the geomean of three internal reference genes: *EF1α, GADPH* and *TUB2*. Data are means ± SE with individual data points plotted (n = 3 pools of approx 60 plants). Statistical differences were determined using a Kruskal-Wallis rank sum test (Asterisks indicate significant differences with the mock control ***$P < 0.001$). **(b)** The length of the bud (mm) at node 3 of 17-day-old *Pisum sativum* plants on a Térèse background measured 7 days after treatment with 10 μL of a solution containing 0 (mock) or 50 μM BA (CK). Data are means ± SE with individual data points plotted (n = 13-24). Statistical differences between the mock and CK treatments for each genotype were determined using Student t-test (* represents means statistically different from the mock treatment $P<0.05$). **(c)** Node 2 buds of 10-day-old *Pisum sativum* wild-type (WT) or *brc1* seedlings on a Cameor background were treated for 6 hrs with 1 mM BA (CK). Expression is represented relative to the WT mock and was normalized against a *GADPH* internal reference gene. Data are means ± SE with individual data points plotted (n = 3-4 pools of 4 plants). **(d)** Node 3 buds of 11 day old *Pisum sativum* wild-type (WT), *rms3* or *smxl7-1* seedlings on a Térèse background were treated for 2 hrs with 0 or 50 μM BA (CK). Expression is relative to the WT mock and was normalized against the geomean of internal reference genes *GADPH* and *EF1α*. Data are means ± SE with individual data points plotted (n=3-6 pools of 6 buds). **(c-d)** Statistical differences between the mock and CK treatments for each genotype were determined using Kruskal-Wallis rank sum test (Asterisks indicate significant differences *$P < 0.05$ and ***$P < 0.001$).

Figure 7. CK increases *D53* transcript levels in rice both in the shoot base and in calli. (a, b) Gene expression in WT rice **(a)** shoot bases and **(b)** calli treated with different concentrations of BA (CK) and harvested at the time points indicated. Expression is represented relative to the 0 μM mock treatment at each time point and was normalized against an *Actin* internal reference gene. Data are means ± SE with individual data points plotted (n = >3 pools of >8 plants). Statistical differences were determined for each time point separately using a one-way ANOVA with a Tukey multiple comparison of means post-hoc test, statistical differences of P<0.05 are represented by different letters.

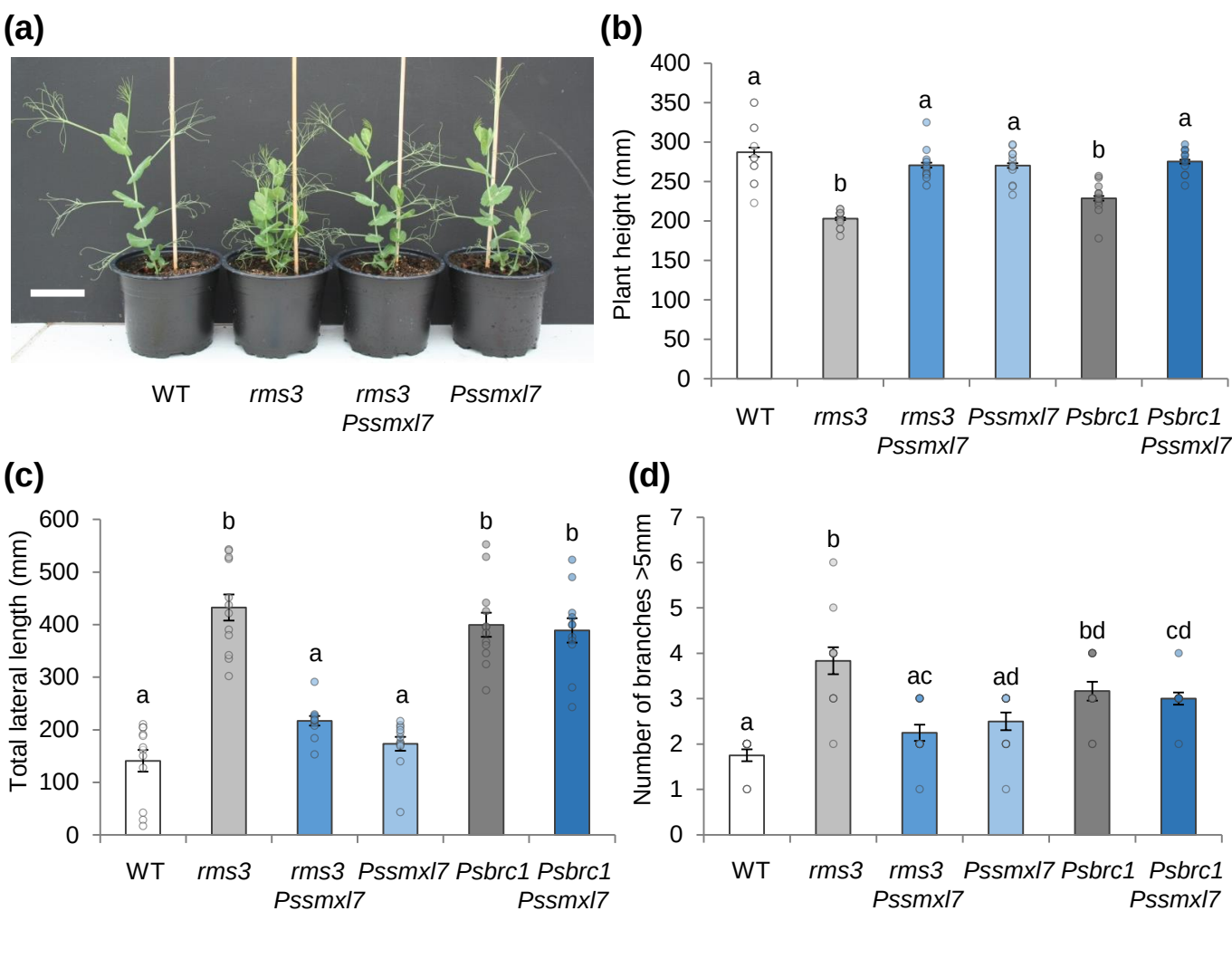
Figure 8. *Pssmxl7* can restore IAA to WT levels in the SL perception mutant *rms3*. IAA levels (ng/g FW) in stem tissue of 21-day-old *Pisum sativum* seedlings on a Térèse background. Data are means ± SE with individual data points plotted (n = 4 pools of 10-12 plants). Statistical differences were determined using a Kruskal-Wallis rank sum test, statistical differences of P<0.05 are represented by different letters.

Figure 9. *Pssmxl7* can partially restore plant height and branching to WT levels in the *Psafb4/5* mutant. (a) Phenotype and quantification of **(b)** plant height (mm) and **(c, d)** total lateral length (mm) of 18-day-old *Pisum sativum* seedlings on a Térèse background. Bar, 5 cm. Data are means ± SE with individual data points plotted (n = 11-12). Statistical differences were determined using a one-way ANOVA with a Tukey multiple comparison of means post-hoc test, statistical differences of P<0.05 are represented by different letters; for **(d)** statistical differences were calculated for each node individually.

Figure 10. Proposed model for the role of PsSMX7 in SL and CK regulation of shoot branching in pea buds.

PsSMXL7 promotes bud outgrowth by inhibiting *PsBRC1* expression to prevent PsBRC1 from repressing bud outgrowth via downstream response genes such as *PsNCED2*. SL inhibits bud outgrowth by targeting PsSMXL7 protein for ubiquitination and degradation. PsSMXL7 negative feedback regulation on *PSMXL7/8* expression results in upregulation of *PSMXL7/8* transcription after SL treatment. PsSMXL7 is also likely involved in feedback regulation of SL biosynthesis by increasing IAA levels in the stem which, through RMS2, increases *RMS1/5* expression. CK likely

promotes bud outgrowth through multiple pathways. Firstly, CK appears to antagonise SL signaling by upregulating *PsSMXL7/8* expression, and downregulating *PsBRC1* expression. Whether CK independently regulates each of these components of the signaling pathway (as shown in the diagram), or *PsBRC1* expression are an indirect result of increased *PsSMXL7/8* expression is unclear. CK also promotes bud outgrowth through a BRC1/SMXL7-independent pathway.


(a)
WT
rms3
rms3 Pssmxl7
Pssmxl7
(b)
Plant height (mm)
400
350
300
250
200
150
100
50
0
a
b
a
a
b
a
WT
rms3
rms3 Pssmxl7
Pssmxl7
Psbrc1
Psbrc1 Pssmxl7
(c)
Total lateral length (mm)
600
500
400
300
200
100
0
a
b
a
a
b
b
WT
rms3
rms3 Pssmxl7
Pssmxl7
Psbrc1
Psbrc1 Pssmxl7
(d)
Number of branches >5mm
7
6
5
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3
2
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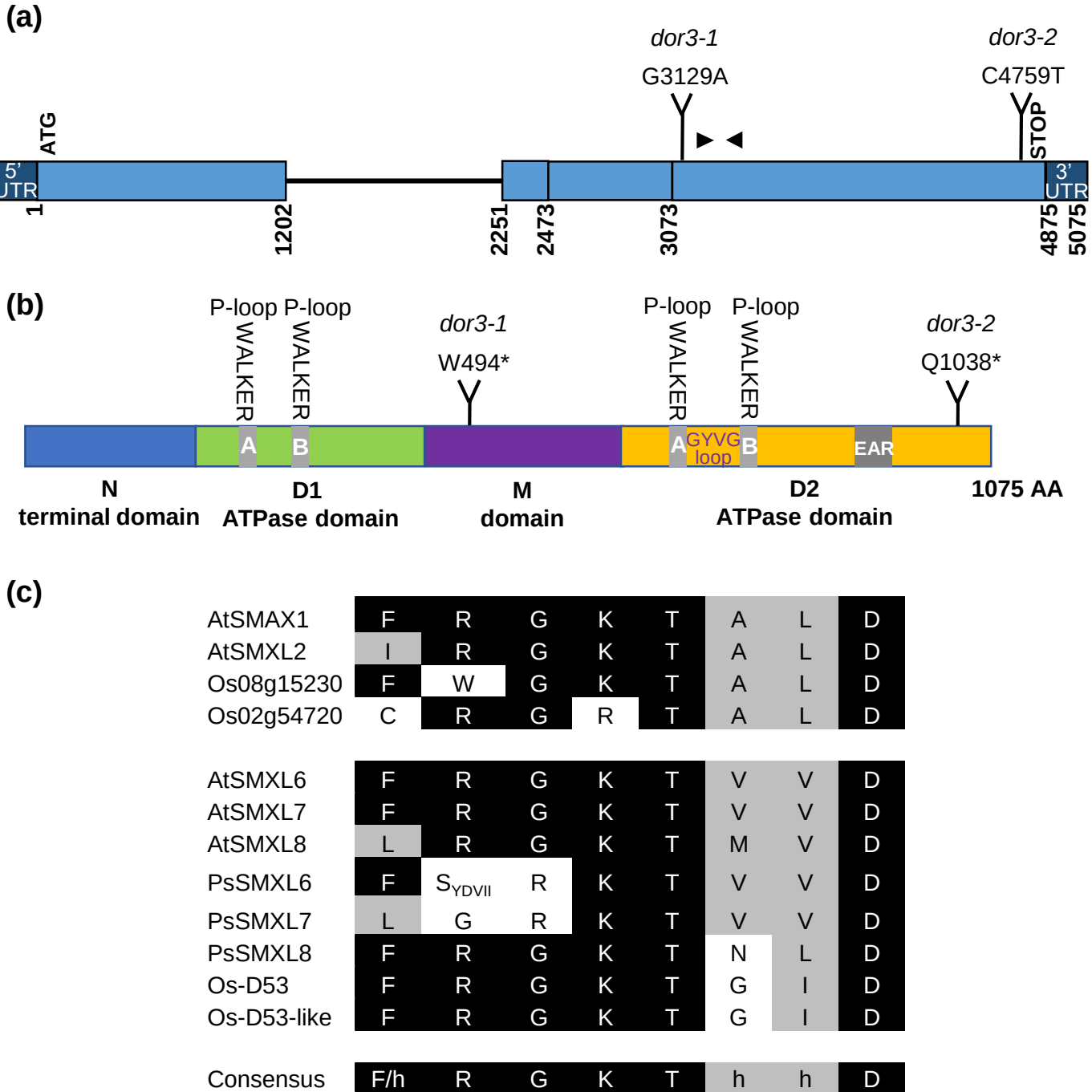
(a)
dor3-1
G3129A
dor3-2
C4759T
ATG
STOP
5' UTR
3' UTR
1
1202
2251
2473
3073
4875
5075
(b)
P-loop
WALKER
A
B
dor3-1
W494*
dor3-2
Q1038*
GYVG loop
EAR
N terminal domain
D1 ATPase domain
M domain
D2 ATPase domain
1075 AA
(c)
AtSMAX1 F R G K T A L D
AtSMXL2 I R G K T A L D
Os08g15230 F W G K T A L D
Os02g54720 C R G R T A L D
AtSMXL6 F R G K T V V D
AtSMXL7 F R G K T V V D
AtSMXL8 L R G K T M V D
PsSMXL6 F S YDVII R K T V V D
PsSMXL7 L G R K T V V D
PsSMXL8 F R G K T N L D
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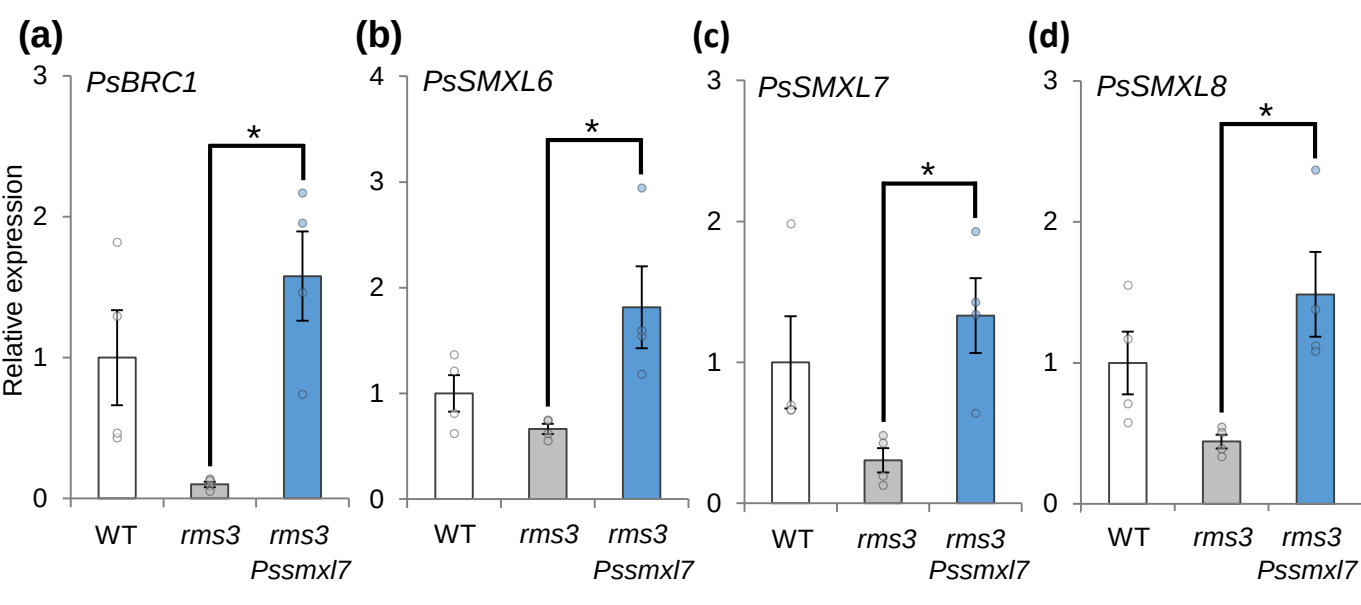
Os-D53-like F R G K T G I D
Consensus F/h R G K T h h D


(a)
PsBRC1
Relative expression
3
2
1
0
*
WT
rms3
rms3 Pssmxl7
(b)
PsSMXL6
4
3
2
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WT
rms3
rms3 Pssmxl7
(c)
PsSMXL7
3
2
1
0
*
WT
rms3
rms3 Pssmxl7
(d)
PsSMXL8
3
2
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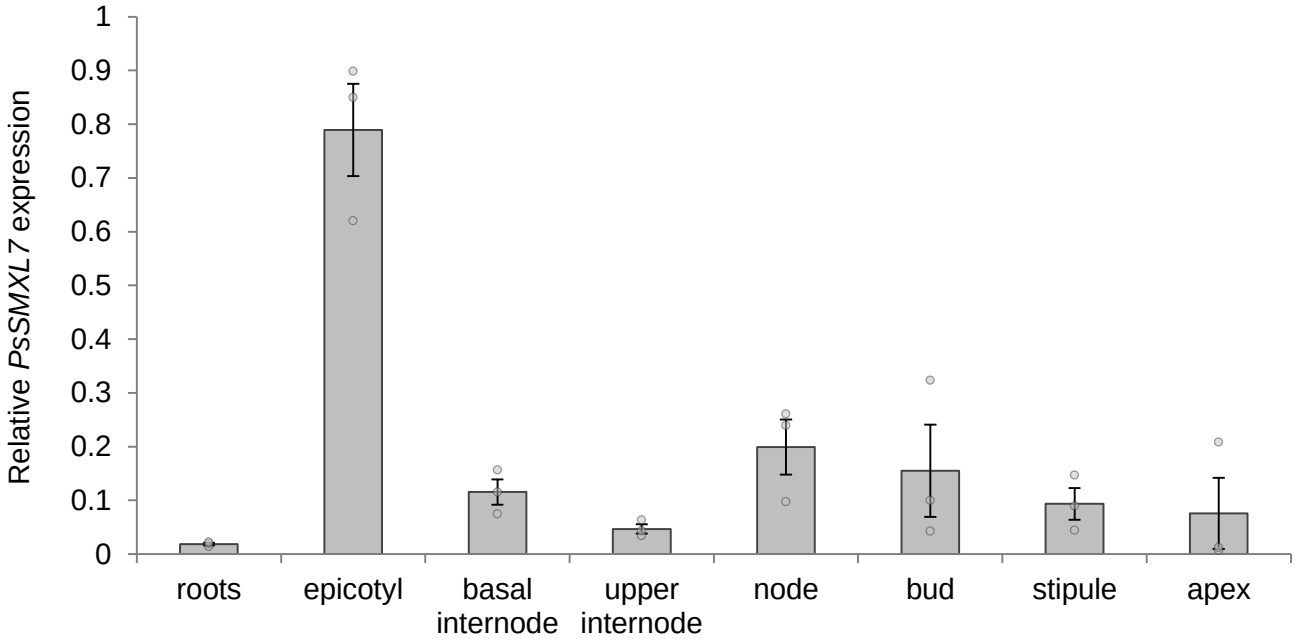
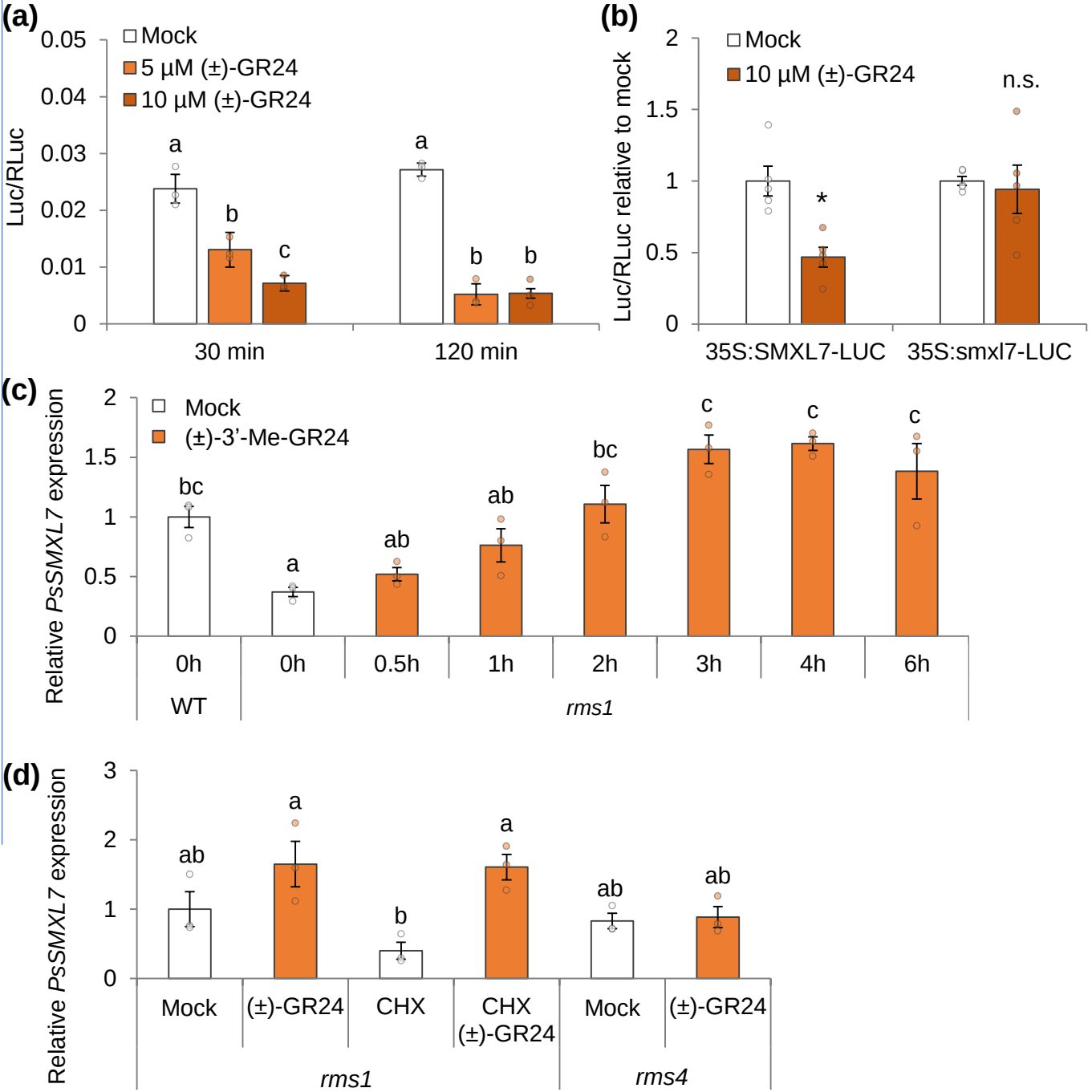
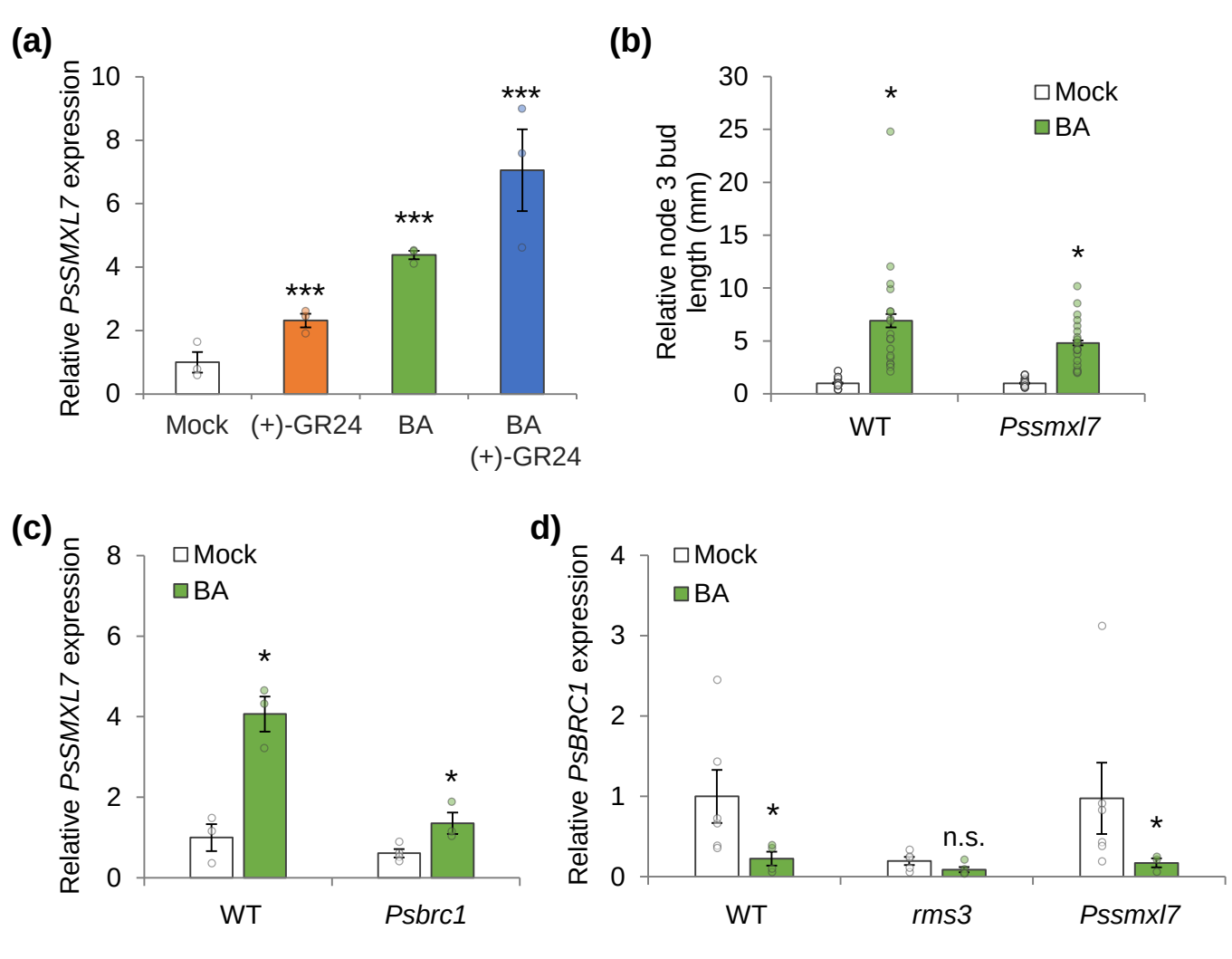
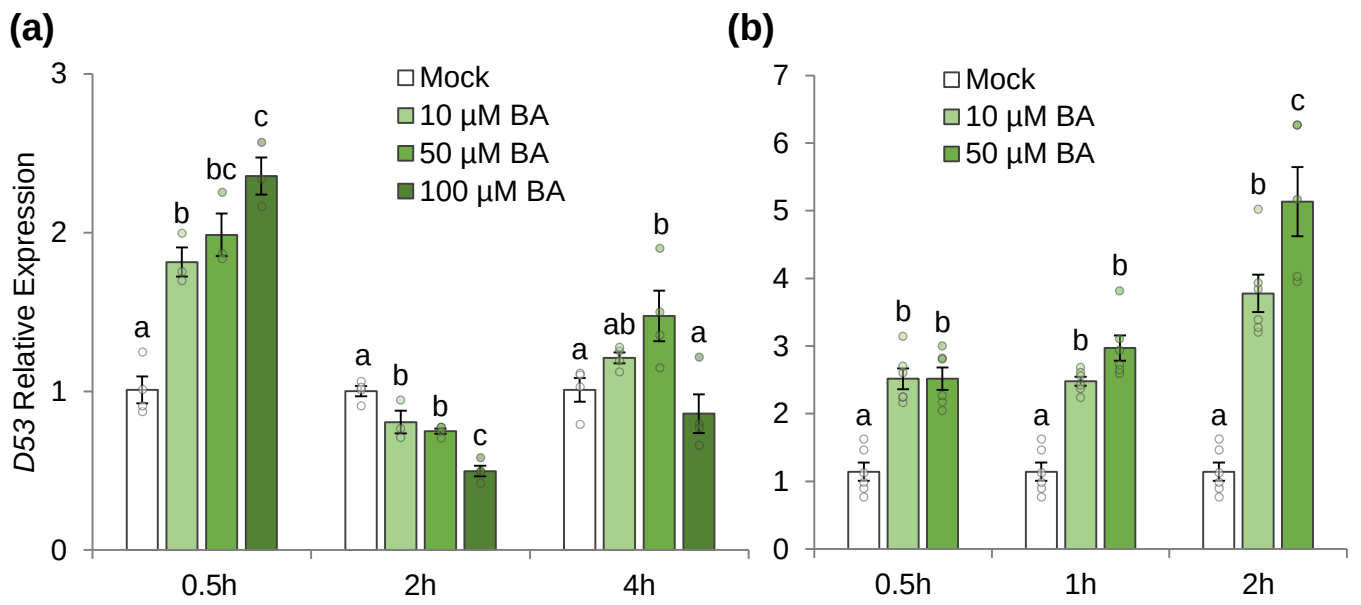
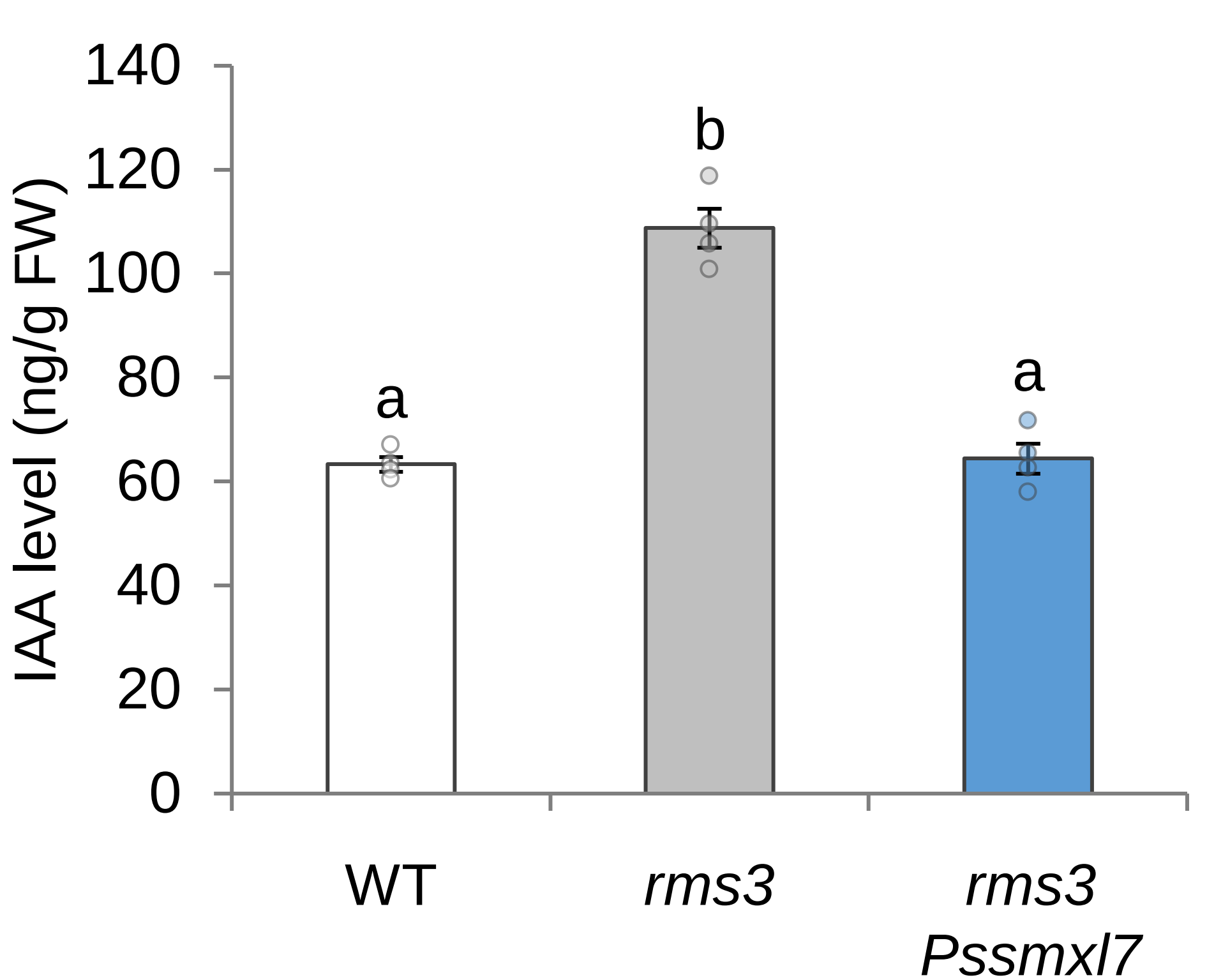
WT
rms3
rms3 Pssmxl7

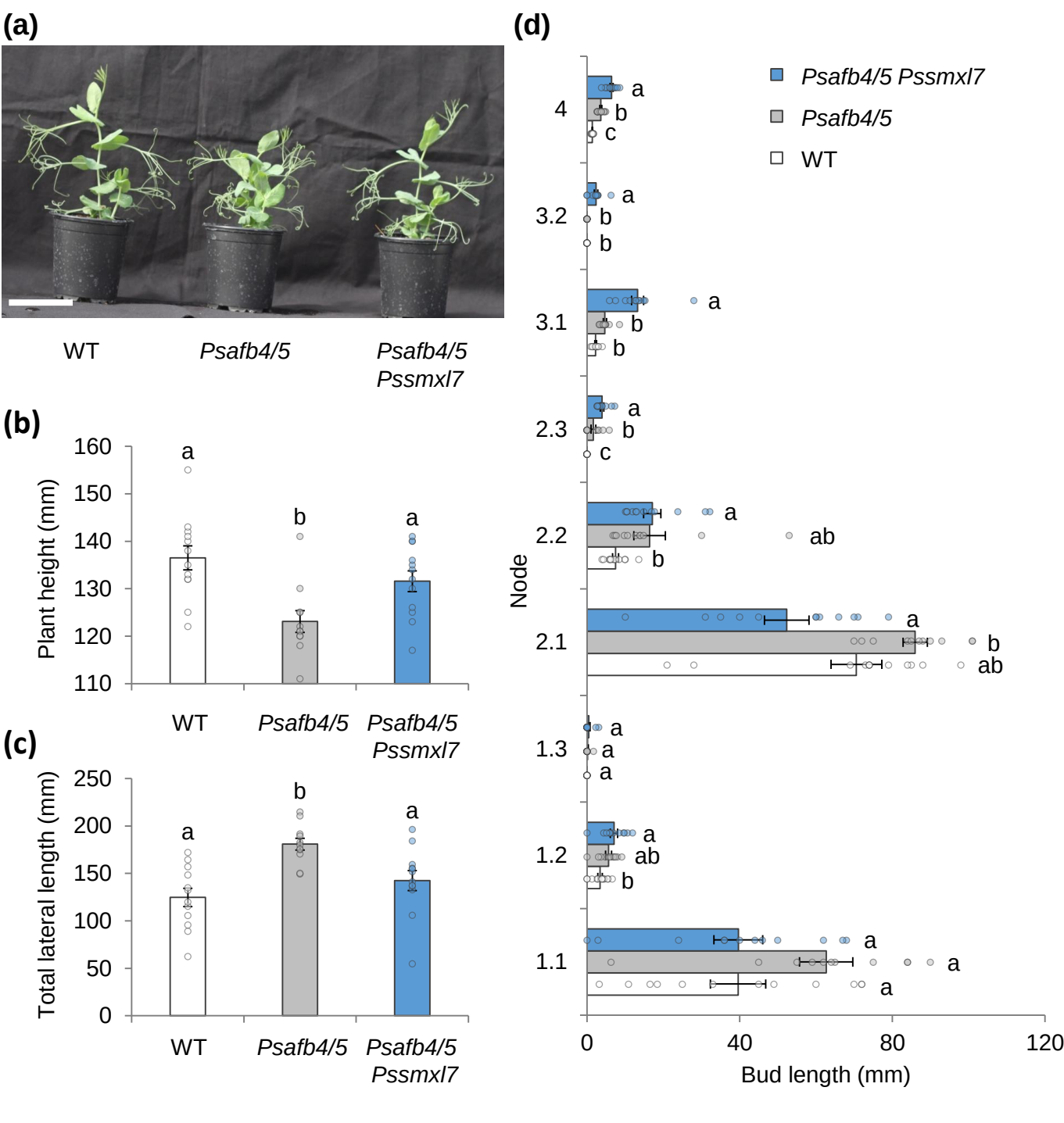

Relative PsSMXL7 expression
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
roots
epicotyl
basal internode
upper internode
node
bud
stipule
apex

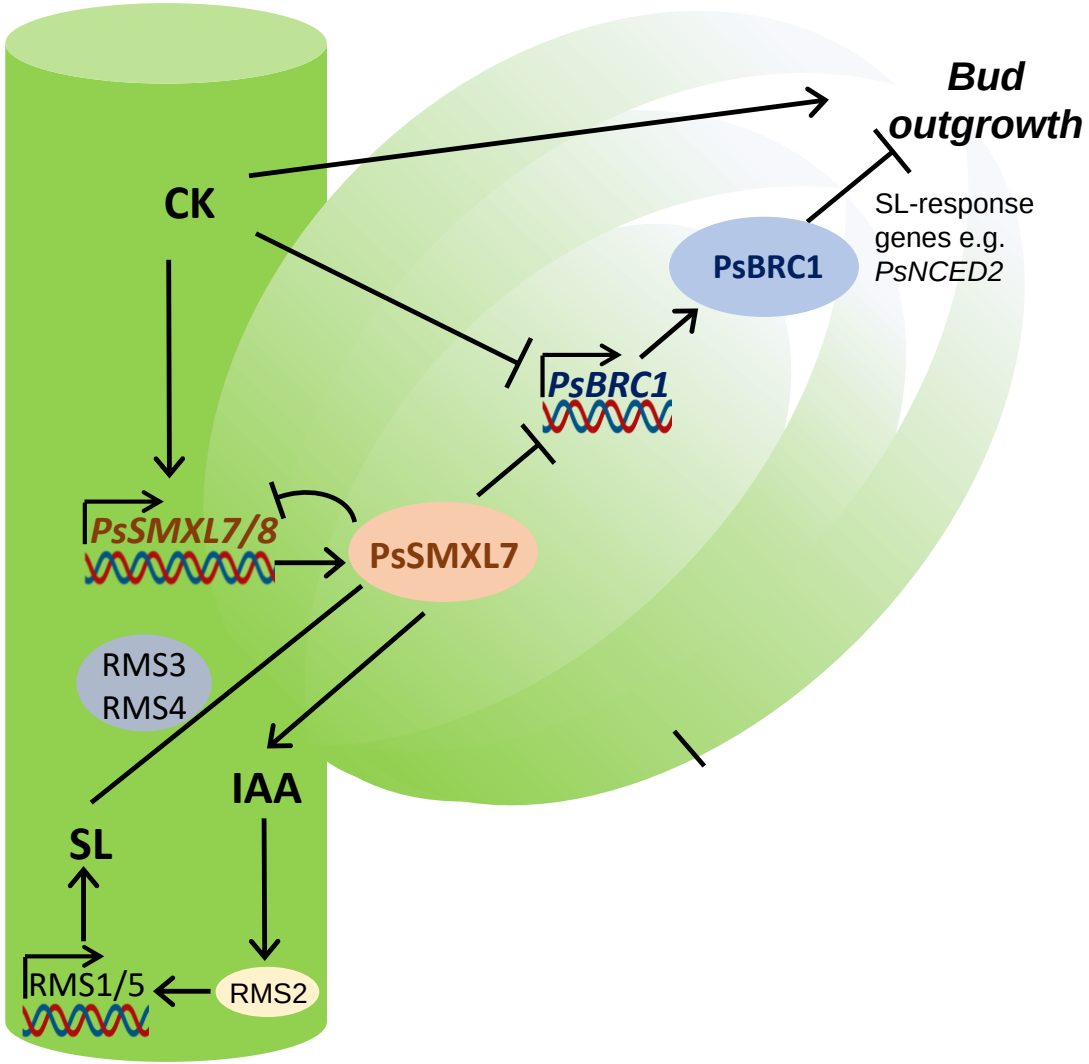

(a)
Mock
5 μM (±)-GR24
10 μM (±)-GR24
Luc/RLuc
0.05
0.04
0.03
0.02
0.01
0
a
b
c
a
b
b
30 min
120 min
(b)
Mock
10 μM (±)-GR24
Luc/RLuc relative to mock
2
1.5
1
0.5
0
*
n.s.
35S:SMXL7-LUC
35S:smxl7-LUC
(c)
Mock
(±)-3'-Me-GR24
Relative PsSMXL7 expression
2
1.5
1
0.5
0
bc
a
ab
ab
bc
c
c
c
0h
WT
0h
0.5h
1h
2h
3h
4h
6h
rms1
(d)
Relative PsSMXL7 expression
3
2
1
0
ab
a
b
a
ab
ab
Mock
(±)-GR24
CHX
CHX
(±)-GR24
Mock
(±)-GR24
rms1
rms4


(a)
Relative PsSMXL7 expression
10
8
6
4
2
0
***
***
***
Mock
(+)-GR24
BA
BA
(+)-GR24
(b)
Relative node 3 bud length (mm)
30
25
20
15
10
5
0
*
*
Mock
BA
WT
Pssmxl7
(c)
Relative PsSMXL7 expression
8
6
4
2
0
Mock
BA
*
*
WT
Psbrc1
(d)
Relative PsBRC1 expression
4
3
2
1
0
Mock
BA
*
n.s.
*
WT
rms3
Pssmxl7


(a)
(b)
D53 Relative Expression
Mock
10 μM BA
50 μM BA
100 μM BA
0
1
2
3
4
5
6
7
a
b
bc
c
ab
0.5h
1h
2h
4h


140
120
100
80
60
40
20
0
IAA level (ng/g FW)
a
b
a
WT
rms3
rms3
Pssmxl7


(a)
WT
Psafb4/5
Psafb4/5 Pssmxl7
(b)
Plant height (mm)
160
150
140
130
120
110
a
b
a
WT
Psafb4/5
Psafb4/5 Pssmxl7
(c)
Total lateral length (mm)
250
200
150
100
50
0
a
b
a
WT
Psafb4/5
Psafb4/5 Pssmxl7
(d)
Psafb4/5 Pssmxl7
Psafb4/5
WT
Node
4
3.2
3.1
2.3
2.2
2.1
1.3
1.2
1.1
a
b
c
a
b
b
a
b
b
a
b
c
a
ab
b
a
b
ab
a
a
a
a
ab
b
a
a
a
0
40
80
120
Bud length (mm)


Bud outgrowth
CK
SL-response genes e.g. PsNCED2
PsBRC1
PsBRC1
PsSMXL7/8
PsSMXL7
RMS3
RMS4
IAA
SL
RMS1/5
RMS2