Annals of Botany **113**: 1037–1045, 2014
doi:10.1093/aob/mcu030, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
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The potential roles of strigolactones and brassinosteroids in the autoregulation of nodulation pathway

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Received: 16 October 2013 Returned for revision: 31 January 2014 Accepted: 12 February 2014 Published electronically: 2 April 2014

• *Background and Aims* The number of nodules formed on a legume root system is under the strict genetic control of the autoregulation of nodulation (AON) pathway. Plant hormones are thought to play a role in AON; however, the involvement of two hormones recently described as having a largely positive role in nodulation, strigolactones and brassinosteroids, has not been examined in the AON process.
• *Methods* A genetic approach was used to examine if strigolactones or brassinosteroids interact with the AON system in pea (*Pisum sativum*). Double mutants between shoot-acting (*Psclv2*, *Psnark*) and root-acting (*Psrdn1*) mutants of the AON pathway and strigolactone-deficient (*Psccd8*) or brassinosteroid-deficient (*lk*) mutants were generated and assessed for various aspects of nodulation. Strigolactone production by AON mutant roots was also investigated.
• *Key Results* Supernodulation of the roots was observed in both brassinosteroid- and strigolactone-deficient AON double-mutant plants. This is despite the fact that the shoots of these plants displayed classic strigolactone-deficient (increased shoot branching) or brassinosteroid-deficient (extreme dwarf) phenotypes. No consistent effect of disruption of the AON pathway on strigolactone production was found, but root-acting *Psrdn1* mutants did produce significantly more strigolactones.
• *Conclusions* No evidence was found that strigolactones or brassinosteroids act downstream of the AON genes examined. While in pea the AON mutants are epistatic to brassinosteroid and strigolactone synthesis genes, we argue that these hormones are likely to act independently of the AON system, having a role in the promotion of nodule formation.

Key words: Autoregulation of nodulation, AON, brassinosteroids, *CLAVATA2*, *NARK*, *Pisum sativum*, *RDN1*, strigolactones.

INTRODUCTION

Legume nodulation occurs as a result of a symbiosis with rhizobia soil bacteria, leading to the formation of novel structures called nodules. Hosted inside the specialized nodule, rhizobia fix atmospheric nitrogen into a form that is accessible to the plant and in exchange are provided with plant carbohydrates (Ferguson *et al*., 2010). Although beneficial, it is essential that the plants balance nitrogen gained with carbon expended, and so nodulation is strictly controlled both locally and systemically through a specialized autoregulation of nodulation (AON) pathway (Reid *et al*., 2011*a*).

Although not fully defined, significant progress has been made in our understanding of the AON pathway (Reid *et al*., 2011*a*; Okamoto *et al*., 2013). Following exposure to rhizobia bacteria, genes are induced that encode mobile CLAVATA3/ESR-related (CLE) peptides (Okamoto *et al*., 2009, 2013; Mortier *et al*., 2010; Lin *et al*., 2011; Reid *et al*., 2011*b*, 2013; Hayashi *et al*., 2012). These peptides move up into the shoot where they are perceived by a leucine-rich repeat receptor-like kinase (LRR RLK) (Krusell *et al*., 2002; Nishimura *et al*., 2002; Searle *et al*., 2003; Schnabel *et al*., 2005). Orthologues of this LRR RLK have been given different names, but we refer to the receptor here as Nodulation Autoregulation Receptor Kinase (NARK), consistent with its name in soybean (Searle *et al*., 2003). Activation of NARK triggers the production of an as yet unidentified shoot-derived factor that is translocated to the roots to inhibit further nodulation (e.g. Delves *et al*., 1986; Lin *et al*., 2010, 2011). Additional receptors, called KLAVIER (KLV) and CLAVATA2 (CLV2), also act in the shoot to control nodule numbers. A model has been proposed in which NARK complexes with CLV2 and/or KLV in the shoot to control nodule numbers in the root (Miyazawa *et al*., 2010; Krussel *et al*., 2011). Mutations in the *NARK*, *KLV* or *CLV2* genes in several legume species lead to a supernodulation phenotype, with a large increase in the number of nodules formed on the roots (Krusell *et al*., 2002, 2011; Nishimura *et al*., 2002; Searle *et al*., 2003; Schnabel *et al*., 2005; Miyazawa *et al*., 2010). As stated above, these genes act in the shoot, as grafting studies have shown that *nark*, *klv* or *clv2* mutant shoots induce supernodulation in wild-type roots (e.g. Delves *et al*., 1986; Sagan and Duc, 1996; Oka-Kira *et al*., 2005).

Autoregulation of nodulation mutants acting in the root that may disrupt induction of the root-to-shoot CLE peptides or perception of the shoot-derived factor have also been identified (e.g. Postma *et al*., 1988; Takahara *et al*., 2013). These include the *nod3* mutant of pea (Postma *et al*., 1988). *NOD3* has recently

been identified as the orthologue of the *Medicago truncatula* gene *RDN1* (Schnabel *et al*., 2011). *RDN1* is expressed in the root vasculature and encodes a protein of unknown function that is a member of a highly conserved gene family unique to green plants, including green algae (Schnabel *et al*., 2011). Grafting studies with both *Mtrdn1* and *Psnod3* mutants have indicated that the supernodulating phenotype is root controlled and that *RDN1* is likely to act very early in AON, in either the generation or transport of the root-to-shoot CLE peptides or alongside this system (Postma *et al*., 1988; Li *et al*., 2009; Schnabel *et al*., 2011; Osipova *et al*., 2012). Indeed, elegant grafting studies by Novak (2010) indicate that an allele of *Psnod3* (*RisfixC*) lacks the root-to-shoot AON signal, as wild-type adventitious roots that formed on wild-type shoots grafted to large supernodulating *Psnod3* roots also displayed a supernodulating phenotype.

Plant hormones have also been implicated in AON signalling (Ferguson and Mathesius, 2003; Ferguson *et al*., 2010; Saur *et al*., 2011; Ryu *et al*., 2012), with good evidence for a role for polar auxin transport (e.g. van Noorden *et al*., 2006; Jin *et al*., 2012). Roles for other hormones such as jasmonic acid, cytokinins and brassinosteroids have also been suggested from studies using a combination of hormone application, hormone measurement and AON mutant studies.

Brassinosteroids are an especially interesting case. Pea mutants defective in brassinosteroid biosynthesis or receptors display a reduction in nodulation, suggesting a positive role for brassinosteroids in nodule development (Ferguson *et al*., 2005). However, brassinosteroids appear to be relatively immobile in pea (Symons and Reid, 2004). Thus, restoration of nodulation in brassinosteroid-deficient pea roots by grafting to wild-type shoots indicates that brassinosteroids may influence nodulation via a shoot-derived signal (Ferguson *et al*., 2005). There is no evidence that this signal is shoot-derived auxin or gibberellins, as grafting did not modify the levels of these hormones (Ferguson *et al*., 2005). This systemic effect of shoot-derived brassinosteroids on nodulation was also seen in soybean application studies, but, interestingly, in these studies, brassinosteroids appeared to act as an inhibitor of nodulation. Exogenous application of a brassinosteroid synthesis inhibitor actually enhanced nodule formation in wild-type soybean, and brassinosteroid application to the shoots suppressed the nodule number in the soybean *nark* mutant but not in wild-type plants (Terakado *et al*., 2005). Clearly, additional studies are required to investigate how brassinosteroids interact with the AON pathway.

The most recent group of plant hormones to be implicated in the control of nodulation is the strigolactones. Strigolactones suppress axillary shoot branching, and roles in other aspects of plant development are currently emerging (Foo and Reid, 2013). Pea mutants disrupted in strigolactone biosynthesis have reduced nodulation, and nodulation can be increased in the mutant by application of the synthetic strigolactone, GR24 (Foo and Davies, 2011). GR24 application can also enhance nodulation in wild-type pea, *Medicago sativa* and *Lotus japonicus* (Soto *et al*., 2010; Foo and Davies, 2011; Liu *et al*., 2013). Suppression of strigolactone biosynthesis gene expression in transgenic *L. japonicus* plants also results in reduced nodulation (Liu *et al*., 2013), indicating a positive role for strigolactones in both determinate (*L. japonicus*) and indeterminate (pea, *M. sativa*) nodule development. Although mobile, strigolactones appear to move only in the direction of root to shoot (Foo *et al*., 2001; Foo and Davies, 2011). Grafting studies indicate that unlike brassinosteroids, strigolactones do not influence nodulation by controlling a shoot-derived factor (Foo and Davies, 2011). Genetic studies indicate that strigolactones may act relatively early in nodule initiation, rather than in nodule organogenesis (Foo *et al*., 2013*a*, *b*), similar to the developmental stage at which the AON pathway is thought to be initiated (Reid *et al*., 2011*a*).

In this study, a genetic approach was used to analyse the interaction between brassinosteroids and strigolactones and the AON pathway in pea. Double mutants were generated containing mutations in elements of the AON pathway (*Psnark*, *Psclv2*, *Psrdn1*; Krusell *et al*., 2002, 2011; Schnabel *et al*., 2011) and either brassinosteroid biosynthesis (*lk*; Nomura *et al*., 2004) or strigolactone biosynthesis (*Psccd8*; Gomez-Roldan *et al*., 2008). The nodulation phenotypes of these double mutants were subsequently assessed. We also monitored strigolactone levels in AON mutants to explore potential links between strigolactone production and the AON pathway.

MATERIALS AND METHODS

Plant material

Crosses were performed with *Pisum sativum* parental lines *lk* (brassinosteroid-deficient, line 212−, on a line 212+ background; Reid, 1986; Nomura *et al*., 2004) or *Psccd8* (strigolactone-deficient, formerly *rms1-2 T*, crossed into a 'Torsdag' background; Foo and Davies, 2011), with *Psnark* (P88, formerly *Pssym29*), *Psclv2* (P64, formerly *Pssym28*) and *Psrdn1* (P79, formerly *Psnod3*) (all supernodulating lines are derived from the parental line 'Frisson'; Duc and Messager, 1989; Sagan and Duc, 1996). Double mutants were selected in the F_2, F_3 or F_4 generations. As the parental AON mutant lines, *Psnark*, *Psclv2* and *Psrdn1* carry the dwarf *le-1* mutation, while *lk* and *Psccd8* are on wild-type (*LE*) backgrounds at this locus, only double mutants that displayed the wild-type *LE* phenotype were selected. Double mutant phenotypes were confirmed by segregation from heterozygous plants already confirmed as homozygous for the other mutation.

Nodulation experiments

Nodulation experiments were carried out as previously described (Foo and Davies, 2011). Briefly, unless otherwise stated, plants were grown under glasshouse conditions in 2 L pots in a 1:1 mixture of dolerite chips and vermiculite topped with vermiculite. Plants were inoculated 7 d after planting with *Rhizobium leguminosarum* bv *viciae* (RLV248) grown in yeast–mannitol broth and received modified Long Ashton solution (Hewitt, 1966) with no nitrogen and 5 mm NaH_2PO_4 weekly. After 35 or 49 d, shoot height, shoot branching and the total number of nodules on each root system were scored. Nodule spacing data were obtained by placing secondary roots on a grid and selecting nodules that intersected the grid. The distance along the root to the next nodule was recorded and the average space between nodules of six plants per genotypes was calculated (note that roots that contained <3 nodules could not

be scored). Root, shoot and nodules were separated and dried at 55 °C to obtain the dry weight. Nodule number is expressed as the number of nodules per g root dry weight for each plant to account for difference in root size, and hence sites for nodule formation, between individual plants.

Strigolactone extraction and measurements

Plants were grown in growth cabinets [20 °C/15 °C day/night under cool-white fluorescent tubes (100 μmol m^{-2} s^{-1}) and an 18 h photoperiod] under semi-sterile conditions to exclude rhizobial bacteria. Pots, seeds and growth trays were sterilized with 70 % ethanol and seeds were planted into 1 L pots containing sterile vermiculite watered with milliQ water. Plants received no nutrients, to induce nitrogen and phosphate starvation, which enhances strigolactone production in pea (Foo *et al*., 2013*a*). When plants were 26 d old, roots were severed from the shoot, washed and weighed. Strigolactones were extracted from root tissue using a modified version of the method reported in Foo and Davies (2011). Following tissue homogenization and overnight extraction in ethyl acetate at 4 °C, samples were filtered and labelled strigolactone standards were added (1 ng each of [6′-$^{2}H_1$]orobanchol, [6′-$^{2}H_1$]orobanchyl acetate and [6′-$^{2}H_1$]fabacyl acetate). Samples were then dried, re-suspended in 0·4 % acetic acid and passed through a 200 mg silica column (Waters Pty Ltd, Australia). The column was washed twice with 15:85 ethyl acetate:hexane and strigolactones were eluted with 45:55 ethyl acetate:hexane. Samples were dried, resuspended in 50:50 acetonitrile:water and detected by UPLC-MS as described by Foo and Davies (2011).

Statistical analysis

For each variable, genotypes were compared by performing one-way analyses of variance (ANOVAs) and post-hoc test (least significant difference) at a threshold of at least $P < 0{\cdot}01$ using the SPSS software package (IBM).

RESULTS

Brassinosteroid-deficient AON double mutants display supernodulation

Brassinosteroid-deficient AON double mutants, *Psnark lk*, *Psclv2 lk* and *Psrdn1 lk*, all displayed classic brassinosteroid-deficient shoot phenotypes that were indistinguishable from the *lk* parent, with smaller, darker leaves, reduced internode lengths and thick stems compared with wild-type or AON parental lines (Fig. 1; Table 1). However, in stark contrast, the roots of *Psnark lk*, *Psclv2 lk* and *Psrdn1 lk* double mutants displayed the classic supernodulating phenotype, indistinguishable from AON parental lines (Fig. 2). Both single-mutant *Psnark*, *Psclv2* and *Psrdn1* and double-mutant *Psnark lk*, *Psclv2 lk* and *Psrdn1 lk* plants all had a significantly increased number of nodules for a given amount of root compared with either wild-type line ('Frisson', 212+) or *lk* (Fig. 2A). Indeed, for all double-mutant plants, the nodule number was not significantly different from that of *Psnark*, *Psclv2* or *Psrdn1* single mutants at the $P < 0{\cdot}01$ level (Fig. 2A).

The nodules that formed on these double-mutant lines were also significantly more closely spaced than observed in wild-type ('Frisson', 212+) or *lk* roots, and this close spacing was indistinguishable from that of AON single mutants (Fig. 2B, C). Interestingly, in contrast to the small nodules formed on AON single-mutant parents, two of the double-mutant plants (*Psclv2 lk* and *Psrdn1 lk*) had somewhat larger individual nodules ($P < 0{\cdot}05$; Table 1). Previous studies have found that brassinosteroid-deficient or insensitive pea mutants had fewer but consistently larger nodules than seen on wild-type plants (Ferguson *et al*., 2005), although that trend was not observed in *lk* mutants in this study (possibly due to the very small number of nodules that could be weighed, as many individual *lk* plants formed no nodules at all; Table 1).

The supernodulating phenotype of brassinosteroid-deficient AON double mutants contrasts with the significantly reduced nodulation observed in brassinosteroid-deficient *lk* single-mutant parents (Fig. 2A; Ferguson *et al*., 2005). Clearly, a dwarf shoot due to brassinosteroid deficiency does not limit nodulation *per se*, as supernodulating brassinosteroid-deficient AON double mutants all displayed a similar dwarf shoot. Further, this maintenance of the supernodulating phenotype in a brassinosteroid-deficient background (i.e. recessive epistasis of the AON mutants over alleles at the *LK* locus) indicates that brassinosteroids do not act downstream of the AON genes examined.

Strigolactone-deficient AON double mutants display supernodulation

We also used a genetic approach to examine whether disrupting strigolactone production influenced the supernodulating phenotype of AON mutants. Strigolactone-deficient AON double mutants, *Psnark ccd8*, *Psclv2 ccd8* and *Psrdn1 ccd8*, all displayed increased shoot branching characteristic of strigolactone-deficient *Psccd8* parents (Fig. 3; Table 2). The number of branches formed on strigolactone-deficient AON double mutants was not significantly different from that on strigolactone-deficient plants on a 'Frisson' background (*Psccd8* on 'Frisson'; Table 2). However, the length of the branches on double mutants was somewhat shorter (Fig. 3), probably due to somewhat reduced root size and increased resource allocation to nodule development in these plants (Table 2).

However, unlike strigolactone-deficient single mutant plants that had significantly fewer nodules than the respective wild types (Fig. 4B; Foo and Davies, 2011), strigolactone-deficient AON double mutants displayed a clear supernodulating phenotype (Fig. 4). Both single-mutant *Psnark*, *Psclv2* and *Psrdn1* and double-mutant *Psnark ccd8*, *Psclv2 ccd8* and *Psrdn1 ccd8* plants all had a significantly increased number of nodules for a given amount of root compared with either wild-type line ('Frisson', 'Torsdag') or *Psccd8* lines (Fig. 4A). Although there were some small differences in the total number of nodules formed on the double mutants compared with the AON single-mutant parents (Fig. 4A), there was no consistent reduction in nodule numbers across the double mutants. Indeed, the characteristic disruption in nodule spacing observed in AON mutants (e.g. Sagan and Gresshoff, 1996; Fig. 4B, C) was observed in all the double-mutant plants investigated here, with a significant decrease in nodule spacing compared with

FIG. 1. Phenotype of 35-day-old wild type ('Frisson', 212+) and various single- and double-mutant combinations of brassinosteroid-deficient *lk* and AON mutants *Psnark*, *Psclv2* and *Psrdn1*. 'Frisson' wild type, *Psnark*, *Psclv2* and *Psrdn1* single-mutant plants carry Mendel's dwarf allele *le-1*, while all other plants carry the wild-type *LE* allele at this locus. Scale bar = 10 cm.

TABLE 1. *Shoot and root phenotypes of 35-day-old wild type ('Frisson', 212+) and various single- and double-mutant combinations of brassinosteroid-deficient* lk *and AON mutants* Psnark, Psclv2 *and* Psrdn1 *(*LE/le *genotype indicated in parentheses)*

Genotype	Leaves expanded	L1–6	Shoot d. wt	Root d. wt	Individual nodule d. wt
'Frisson' (*le*)	8·25 ± 0·31^{a}	62·3 ± 3·6^{a}	188·2 ± 8·8^{a}	52·6 ± 2·9^{a}	0·18 ± 0·06^{ab}
Psnark (*le*)	8·58 ± 0·27^{a}	42·8 ± 2·1^{b}	67·9 ± 10·4^{b}	25·0 ± 2·3^{b}	0·06 ± 0·01^{a}
Psclv2 (*le*)	8·08 ± 0·15^{a}	68·0 ± 4·2^{a}	117·9 ± 5·4^{c}	32·8 ± 2·8^{b}	0·04 ± 0·01^{a}
Psrdn1 (*le*)	7·08 ± 0·33^{a}	52·7 ± 0·8^{a}	121·9 ± 10·4^{c}	38·1 ± 2·8^{ab}	0·03 ± 0·01^{a}
212+ (*LE*)	8·75 ± 0·21^{a}	184·5 ± 3·8^{c}	266·9 ± 7·5^{d}	92·5 ± 5·9^{c}	0·17 ± 0·03^{ab}
lk (*LE*)	5·83 ± 0·17^{b}	38·5 ± 2·4^{b}	66·8 ± 6·2^{b}	23·9 ± 3·9^{b}	0·11 ± 0·03^{ab}
Psnark lk (*LE*)	7·75 ± 1·2^{a}	38·2 ± 6·3^{b}	85·4 ± 9·9^{bc}	26·68 ± 4·2^{b}	0·07 ± 0·01^{a}
Psclv2 lk (*LE*)	7·92 ± 0·93^{a}	39·7 ± 7·4^{b}	60·37 ± 8·4^{bc}	18·9 ± 1·1^{b}	0·23 ± 0·05^{ab}
Psrdn1 lk (*LE*)	7·83 ± 0·77^{a}	39·3 ± 2·2^{b}	87·8 ± 7·9^{b}	24·0 ± 2·6^{b}	0·13 ± 0·02^{ab}

The number of leaves expanded, length of shoot from node 1 to 6 (L1–6) and shoot, root and individual nodule dry weight (mg) are indicated.
Values within a column with different letters indicate significant differences (ANOVA, $P < 0·01$).
Values are the mean ± s.e. ($n = 5–6$).

wild-type ('Frisson', 'Torsdag') or *Psccd8* roots (Fig. 4B). This phenotype was almost indistinguishable from the AON single-mutant parents (Fig. 4B, C). This is in contrast to the small, but not significant, increase in nodule spacing observed in *Psccd8* mutants compared with the respective wild-type parents (Fig. 4B).

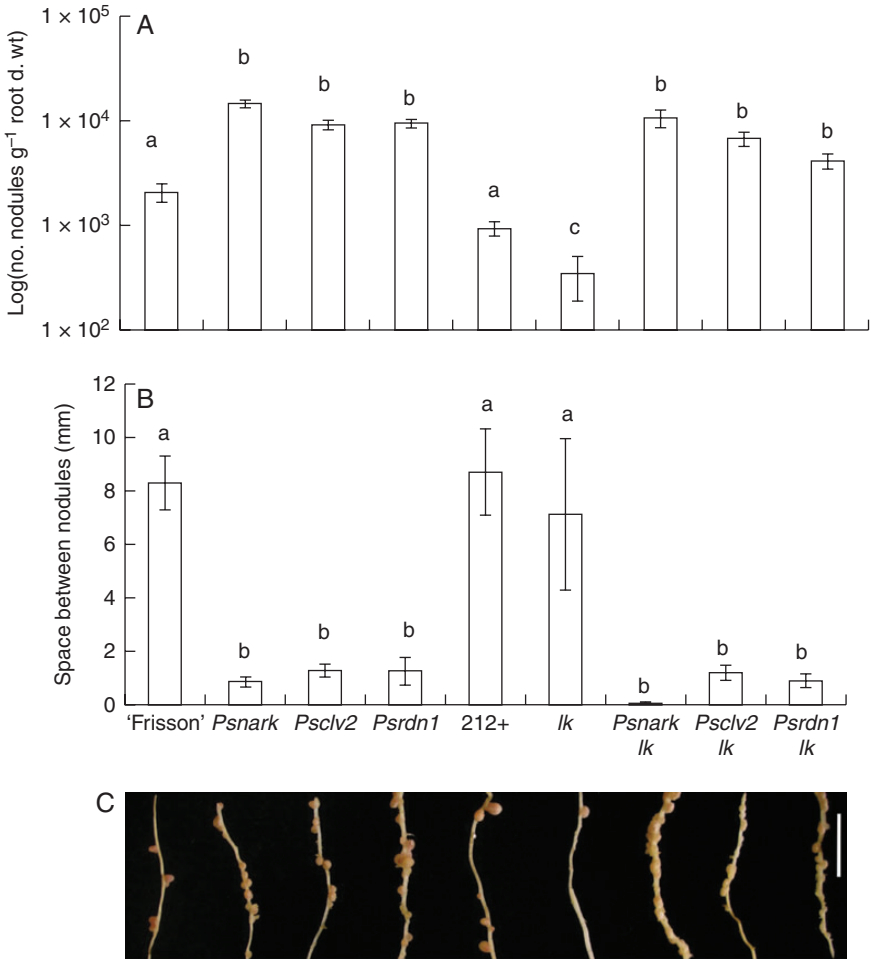

FIG. 2. Nodulation phenotype of 35-day-old wild type ('Frisson', 212 +) and various single- and double-mutant combinations of brassinosteroid-deficient *lk* and AON mutants *Psnar*k, *Psclv2* and *Psrdn1*. (A) Log of number of nodules per gram dry root weight, (B) space between nodules and (C) photographs of nodules on secondary roots (tertiary roots have been removed); the scale bar is 1 cm. Different letters above bars indicate significant differences between genotypes (ANOVA, $P < 0{\cdot}01$). For A and B, values are the mean ± s.e. ($n = 6–7$).

Strigolactone levels in AON mutants

The double-mutant studies outlined above indicate that strigolactones are not essential for the supernodulating phenotype. However, given the strong regulation of strigolactone production by nitrogen and phosphate deficiency (e.g. Foo *et al*., 2013*a*; Yoneyama *et al*., 2012), and the proposed roles for elements of the AON pathway in the control of nodulation under nitrogen and phosphorus limitation (Carrol *et al*., 1985; Sagan and Duc, 1996; Reid *et al*. 2011*a*, *b*; Foo *et al*., 2013*b*), we investigated strigolactone production in the AON mutants. Plants were grown in the absence of rhizobia and no plants formed nodules (data not shown). This was important in case nodules themselves produce strigolactones, which would influence the results.

The level of the three major strigolactones found in pea root tissue, orobanchol, orobanchyl acetate and fabacyl acetate, was similar in wild-type ('Frisson'), *Psnark* and *Psclv2* roots (Fig. 5). However, there was a significant, almost 2-fold increase in all three strigolactones in the root tissue of *Psrdn1* mutant plants compared with wild-type roots.

DISCUSSION

In pea, both strigolactones and brassinosteroids have been shown to have a positive effect on nodule initiation (Ferguson *et al*., 2005; Foo and Davies, 2011), although the exact nature of their influence on nodulation is still emerging. This study indicates that neither brassinosteroids nor strigolactones are likely to play a major role in the AON pathway, since double mutants deficient in either of these hormones and a shoot- and/or root-acting element of AON exhibit a supernodulation phenotype.

In most cases, the roots of the double-mutant plants generated were indistinguishable from those of their single-mutant AON parents, on the basis of elevated nodule number and reduced nodule spacing (Figs 2 and 4). This epistasis of AON mutations to either brassinosteroid or strigolactone deficiency can be interpreted in one of two ways. A classical genetic interpretation would be that strigolactones and brassinosteroids act earlier in the nodulation pathway than the AON system, and hence disruption of the synthesis of either hormone results in no phenotype in an AON mutant. However, given that disruption of the AON system is known to result in a qualitative shift in nodulation

FIG. 3. Phenotype of 49-day-old wild type ('Frisson', 'Torsdag') and various single- and double-mutant combinations of strigolactone-deficient *Psccd8* and AON mutants *Psnark*, *Psclv2* and *Psrdn1*. Arrows indicate shoot branches. 'Frisson' wild type, *Psnark*, *Psclv2* and *Psrdn1* single-mutant plants carry Mendel's dwarf allele *le-1*, while all other plants carry the wild-type *LE* allele at this locus. Scale bar = 10 cm.

TABLE 2. *Shoot and root phenotypes of 49-day-old wild type ('Frisson', 'Torsdag') and various single- and double-mutant combinations of strigolactone-deficient* Psccd8 *and AON mutants* Psnark, Psclv2 *and* Psrdn1 *(*LE/le *genotype indicated in parentheses)*

Genotype	No. of branches	Leaves expanded	L1–6	Shoot d. wt	Root d. wt	Individual nodule d. wt
'Frisson' (*le*)	0^{a}	$13 \pm 0{\cdot}3^{a}$	74 ± 3^{a}	580 ± 62^{a}	86 ± 9^{a}	$0{\cdot}13 \pm 0{\cdot}02^{a}$
Psnark (*le*)	0^{a}	$10{\cdot}7 \pm 0{\cdot}3^{b}$	54 ± 4^{a}	200 ± 44^{a}	36 ± 6^{a}	$0{\cdot}05 \pm 0{\cdot}01^{a}$
Psclv2 (*le*)	0^{a}	$12{\cdot}7 \pm 0{\cdot}5^{a}$	65 ± 2^{a}	349 ± 4^{a}	58 ± 4^{a}	$0{\cdot}11 \pm 0{\cdot}02^{a}$
Psrdn1 (*le*)	$0{\cdot}3 \pm 0{\cdot}3^{a}$	$12{\cdot}8 \pm 0{\cdot}2^{a}$	57 ± 2^{a}	375 ± 20^{a}	49 ± 3^{a}	$0{\cdot}07 \pm 0{\cdot}01^{a}$
'Torsdag' (*LE*)	0^{a}	14 ± 0^{a}	194 ± 16^{b}	1012 ± 79^{b}	176 ± 21^{bc}	$0{\cdot}36 \pm 0{\cdot}05^{b}$
Psccd8 (*LE*)	$9{\cdot}5 \pm 0{\cdot}2^{b}$	15 ± 0^{c}	205 ± 7^{bc}	1178 ± 58^{b}	229 ± 41^{c}	$0{\cdot}27 \pm 0{\cdot}02^{b}$
Psccd8 on 'Frisson' (*LE*)	$4{\cdot}2 \pm 0{\cdot}3^{bc}$	13 ± 0^{a}	206 ± 5^{bc}	1242 ± 53^{b}	120 ± 13^{ab}	$0{\cdot}29 \pm 0{\cdot}02^{b}$
Psnark ccd8 (*LE*)	$5{\cdot}3 \pm 1{\cdot}2^{c}$	$13{\cdot}6 \pm 0{\cdot}9^{a}$	241 ± 7^{c}	1102 ± 131^{b}	60 ± 6^{a}	$0{\cdot}08 \pm 0{\cdot}01^{a}$
Psclv2 ccd8 (*LE*)	$3{\cdot}2 \pm 0{\cdot}3^{bc}$	$15 \pm 0{\cdot}3^{ac}$	190 ± 18^{b}	680 ± 21^{ab}	68 ± 9^{a}	$0{\cdot}03 \pm 0{\cdot}01^{a}$
Psrdn1 ccd8 (*LE*)	$4{\cdot}5 \pm 0{\cdot}6^{bc}$	$13{\cdot}5 \pm 0{\cdot}2^{a}$	234 ± 9^{c}	845 ± 79^{b}	46 ± 5^{a}	$0{\cdot}11 \pm 0{\cdot}01^{a}$

The number of leaves expanded, number of shoot branches (>3 mm) and shoot, root and individual nodule dry weight (mg) are given.
Values within a column with different letters indicate significant differences (ANOVA, $P < 0{\cdot}01$).
Values are the mean ± s.e. ($n = 5–6$).

(supernodulation; in this study up to a 7-fold increase in nodule number), while strigolactone or brassinosteroid deficiency results in only a relatively small quantitative change in nodulation (in this study an approx. 50 % decrease in nodule number), there is a second way to interpret this data. If strigolactones and brassinosteroids influence nodulation independently of the AON pathway, the qualitative shift to a supernodulaing phenotype in an AON mutant may mask any small, quantitative effects of strigolactone or brassinosteroid deficiency in a double-mutant plant. Regardless of which interpretation is correct, the data certainly show that the strigolactones and brassinosteroids do not act further down the AON pathway than the *PsNARK*, *PsCLV2* and *PsRDN1* genes.

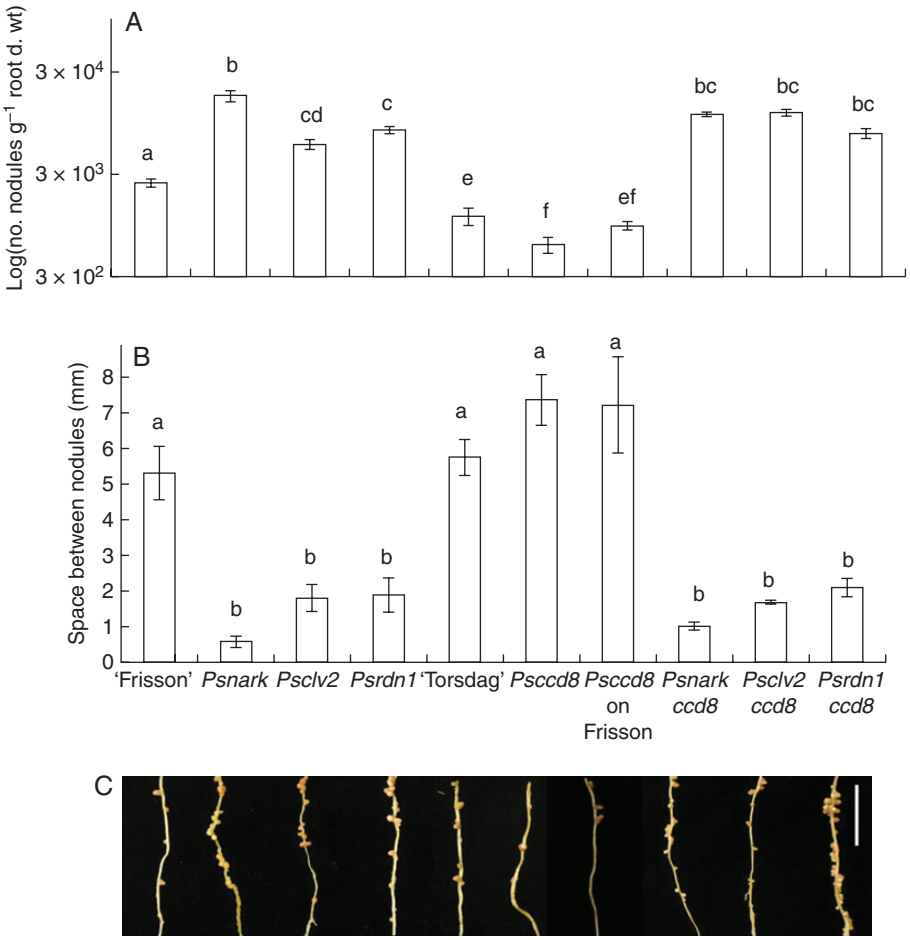

FIG. 4. Nodulation phenotype of 49-day-old wild type ('Frisson', 'Torsdag') and various single- and double-mutant combinations of strigolactone-deficient *Psccd8* and AON mutants *Psnark*, *Psclv2* and *Psrdn1*. (A) Log of number of nodules per gram dry root weight, (B) space between nodules and (C) photographs of nodules on secondary roots (tertiary roots have been removed); the scale bar is 1 cm. Different letters above bars indicate significant differences between genotypes (ANOVA, $P < 0{\cdot}01$). For A and B, values are the mean ± s.e. ($n = 5–6$).

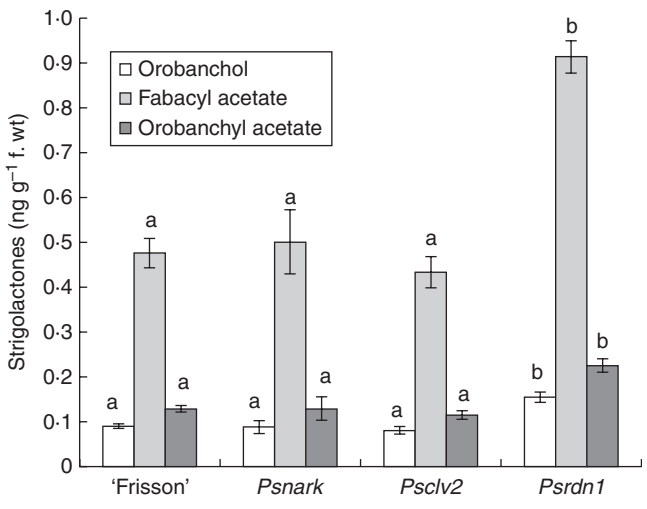

FIG. 5. Strigolactone levels in the root tissue of 21-day-old wild type ('Frisson') and AON mutant lines, *Psnark*, *Psclv2* and *Psrdn1*. Different letters above bars indicate significant differences between genotypes (separate ANOVAs were carried out on each strigolactone, $P < 0{\cdot}01$). Values are the mean ± s.e. ($n = 4$).

An independent role for strigolactones and brassinosteroids in nodulation is consistent with previous studies examining the role of gibberellins in the AON pathway (Ferguson *et al.*, 2011). As observed for double mutants in this study, gibberellin-deficient AON double mutants exhibited a supernodulation phenotype similar to their supernodulating AON parents. However, it is interesting to note that the large number of nodules that formed on gibberellin-deficient AON double mutants were aberrant in structure and appeared to be arrested in development, indicating an important role for gibberellins in nodule organogenesis (Ferguson *et al.*, 2005, 2011). This is in contrast to the fully formed nodules observed on strigolactone- and brassinosteroid-deficient single- or double-mutant plants (Figs 2C and 4C), consistent with previous studies suggesting that these hormones are likely to be involved in nodule initiation but appear less critical for nodule organogenesis (Ferguson *et al.*, 2005; Foo and Davies, 2011; Foo *et al.*, 2013*a*).

The observation that strigolactone levels are elevated in the roots of *Psrdn1* single mutants but not shoot-acting elements of the AON pathway (Fig. 5) is intriguing. The action of the RDN1 protein is still unknown, but studies indicate that it acts

in the vasculature of the root to induce early AON responses either downstream of or at the level of root-derived CLE peptides (Li *et al*., 2009; Schnabel *et al*., 2011; Osipova *et al*., 2013). It is possible that RDN1, but not the AON pathway more generally, normally suppresses strigolactone production in the root. The elevated strigolactones do not appear to contribute to elevated nodulation in *Psrdn1*, as *Psrdn1 ccd8* double mutants had a similar number of nodules to the *Psrdn1* parents (Fig. 4). However, given that strigolactones appear to play a small role in some aspects of root development (e.g. Ruyter-Spira *et al*., 2011), it would be interesting to explore whether the altered root development observed in *Psrdn1* mutant plants (Schnabel *et al*., 2011) may be in part due to altered strigolactone levels.

ACKNOWLEDGEMENTS

We wish to thank A/Professor Noel Davies for assistance with strigolactone measurements, Anthony Cummings, Tiernan O'Rourke, Shelley Urquart, Nicole Bezemer, Helen Paul, Ian Cummings, Michelle Lang and Tracy Winterbottom for technical support, and Professor Koichi Yoneyama (Utsunomiya University) and Dr Kohki Akiyama (Osaka Prefecture University) for their kind gifts of deuterated strigolactone standards. This work was supported by funding from the Australian Research Council.

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