Plant Growth Regulation (2018) 85:231–242
https://doi.org/10.1007/s10725-018-0387-3

ORIGINAL PAPER

Both epiphytic and endophytic strains of *Rhodococcus fascians* influence transporter gene expression and cytokinins in infected *Pisum sativum* L. seedlings

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Received: 12 September 2017 / Accepted: 9 March 2018 / Published online: 15 March 2018

Abstract
Some strains of the soil bacterium *Rhodococcus fascians* maintain an epiphytic life style while others become endophytic. Virulent, endophytic strains cause multiple shoot growth and inhibit root growth of seed-inoculated *Pisum sativum* L. We were interested in assessing, at the molecular level, the impact of strains of contrasting niche on the emerging shoots and roots of inoculated seeds. The presence of *R. fascians* was monitored microscopically, endogenous cytokinin and chlorophyll levels were measured, and the expression of genes monitored by RT-qPCR. The expression of the pea sugar transporter genes (*SWEET* and *SUT*), amino acid (*AAP*) transporters and cell wall invertase gene family members, as well as expression of plant and bacterial cytokinin biosynthesis (*IPT*), activation (*LOG*) and degradation (*CKX*) genes were monitored. Both the virulent strain and the epiphytic strain affected the expression of the transporter genes, with less obvious differences between the strains on the shoot compared with the effect on the root. Strong expression of the *R. fascians* genes, *RfIPT, RfLOG* and *RfCKX*, in pea seedlings at 15 days post inoculation was mirrored by increased expression of transporter gene family members in the plant. However, the elevated levels of isopentenyl adenine-type and zeatin-type cytokinins were not consistently associated with the virulent strain. In conclusion, while both the virulent strain and the epiphytic strain impacted the expression of transporter genes in the shoots and roots, only the virulent strain affected morphology. The inhibited root growth, the greening of the roots, and the expression of the pea response regulators in the infected roots are indicative of a response to cytokinin, but a role for the 'classical' cytokinins as virulence determinants was not established.

Keywords Amino acid transporter · Cell wall invertase · Cytokinin · Pea · *Rhodococcus fascians* · Sucrose transporter · Sugar Will Eventually be Exported Transporter (SWEET)

Introduction

Multiple strains of the soil bacterium *Rhodococcus fascians* maintain an epiphytic life style while others become endophytic and pathogenic (Savory et al. 2017). Infection by pathogenic strains of *R. fascians* causes leaf deformation, fasciation, leafy galls and the formation of witches' broom in a wide range of monocot and dicot plants (Vereecke et al. 2000, 2003), as well as the inhibition of root growth as shown in peas (Eason et al. 1995; Dhandapani et al. 2017) and arabidopsis (Francis et al. 2016).

To maintain either an epiphytic or endophytic life style, bacteria need to source both carbon- and nitrogen-containing metabolites from the plant (Bezrutczyk et al. 2018). Both an epiphytic and a pathogenic strain of *R. fascians* were shown to affect expression of sugar and amino acid transporters in inoculated pea cotyledons (Dhandapani et al. 2017). The effect of the pathogenic strain of *R. fascians* on the cotyledons of germinating peas was to retain these as a sink for the pathogen as opposed to being used to exhaustion as a source for the growing seedling (Dhandapani et al. 2017). Additionally, Dhandapani et al. (2017) suggested that the interaction

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between cytokinins, cell wall invertases (CWINV) and the sugar transporters known as Sugar Will Eventually be Exported Transporters (SWEET) may lead to the axillary bud outgrowth observed in germinating peas infected with the pathogenic strain.

That the cytokinins have a role in *R. fascians*-induced symptoms is strongly supported by the positive correlation in multiple strains between the presence of a plasmid and the genes for cytokinin biosynthesis (isopentenyl transferase-*IPT*), cytokinin activation (*LOG*), and cytokinin degradation (*CKX*) and virulence. Avirulent strains lack such a plasmid (Gális et al. 2005a; Stange et al. 1996; Dhandapani et al. 2017; Savory et al. 2017). Further, Depuydt et al. (2009) suggested that the over-representation of genes involved in cytokinin perception, signal transduction and homeostasis in microarray data from *Arabidopsis thaliana* infected with *R. fascians* supported the central role of cytokinin as a virulence determinant.

Crespi et al. (1992) showed that the *fas* operon of the linear virulence plasmid pFid188 from *R. fascians* strain 188 consisted of six genes most of which have since been shown to code for enzymes involved in cytokinin biosynthesis and metabolism (Crespi et al. 1992, 1994; Pertry et al. 2010). Isopentenyl transferase (*IPT*) is encoded by the *fasD* gene of *R. fascians; fasE* is homologous to the plant *CKX* gene which acts to degrade cytokinins; *fasF* is homologous to the plant Lonely Guy (*LOG*) which directly converts the products of IPT to active free bases (Kurakawa et al. 2007; Kuroha et al. 2009). Additionally, two SAM-dependent methyl transferases (MT1 and MT2) are located on the *fas* locus upstream of the *fas* genes: the products of MT2 are converted to methylated cytokinins by Fas4 (FasD) (Radhika et al. 2015).

There are conflicting hypotheses regarding the efficacy of *R. fascians*-produced cytokinins and disease symptoms, as the leafy symptoms would normally indicate elevated cytokinin levels (Morris 1987), as would the inhibition of root growth (Werner et al. 2003). Pertry et al. (2010) suggested the continuous presence of cZ and 2MeScZ in *Arabidopsis* infected with *R. fascians* virulent strain D188 enabled continuous tissue proliferation, due to the inefficient metabolism of these compounds by CKX, although neither of these cytokinins was detected in tobacco leafy galls (Pertry et al. 2009). Creason et al. (2014), on the other hand, suggested that only one cytokinin type (the iP-type) was necessary for disease symptoms, a statement somewhat supported by Dhandapani et al. (2017) in *Rhodococcus*-infected cotyledons, while Radhika et al. (2015) suggest that *R. fascians*-produced methylated cytokinins are the key virulence determinants.

Here we report the impact of strains of contrasting niche on the emerging shoots and roots of inoculated seeds. We were interested if the effect of *R. fascians* on the expression of both sugar and amino acid transporters continued as the roots and shoots of inoculated peas developed, and whether the levels of the iP-type cytokinins were elevated in both the shoots and roots of peas inoculated with the pathogenic strain, as would be expected if only the iP-type of cytokinin was needed for infection (Creason et al. 2014).

Materials and methods

Plant material, *Rhodococcus fascians* inoculation and sampling

Pea (*Pisum sativum*) var. Bohatyr seeds were obtained from the Institute for Plant and Food Research, Christchurch, New Zealand. The pea seeds were surface sterilised and inoculated with *R. fascians* pathogenic strain 602, which contains a linear virulence plasmid, and a plasmid-free avirulent strain, 589, as described in Dhandapani et al. (2017).

Six seeds were placed in sterilised 500 ml containers with 0.6% (w/v) agar and 10% (w/v) Hoagland's mineral salts solution (Lawson et al. 1982) and germinated at 22 °C with a 16 h photoperiod in a growth room. Samples of separated shoots and roots from 5 to 35 days post inoculation (dpi) were either immediately immersed in liquid nitrogen and then stored at − 80 °C for gene expression studies, or fixed in FAA (10% formaldehyde:5% acetic acid:50% alcohol) for light microscopy, and cryopreserved for scanning electron microscopy. For each sampling, tissues from five plant samples were collected from each of the three treatments: the avirulent and virulent strain-inoculated peas, and the mock-inoculated controls.

Chlorophyll estimation

The chlorophyll content of shoot and root samples was measured from 5 to 35 dpi by immersing 0.1 g FW in 1 ml dimethylformamide overnight at 4 °C, and using a Nanodrop spectrophotometer to determine the absorbance at 664 and 667 nm as described in Evans et al. (2012).

Light microscopy

A modified procedure of Carletom and Druvy (1957) for fixation, dehydration, embedding and microtoming was followed. The detailed protocol is described in Dhandapani et al. (2017).

RNA isolation and cDNA synthesis

Total RNA was extracted from pea tissues (shoot and root) with the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol and as

explained by Dhandapani et al. (2017). The concentration and integrity of isolated total RNA were assessed using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific Inc.) and 1% (w/v) agarose gel electrophoresis. The extracted RNA was converted to cDNA by reverse transcription, diluted fivefold with TE buffer and stored at − 20 °C. The cDNA quality was validated by RT-qPCR assay by using two reference genes and a target gene (Dhandapani et al. 2017).

Genes of interest and real-time reverse transcription quantitative PCR (RT-qPCR)

Gene isolation, sequence analysis and identification are described in Dhandapani et al. (2017). The detailed phylogenetic analysis of the cytokinin (*PsIPTs, PsLOGs, PsCKXs* and *PsRRs*) gene family members and transporter (*PsSUTs, PsAAPs, PsCWINVs* and *PsSWEETs*) gene family members has been published in Dhandapani et al. (2017). Previously these were aligned using the ClustalX program (Thompson et al. 1997) and MEGA4 (Tamura et al. 2007).

Primers were designed which were unique and specific for the genes of interest based on *P. sativum* transcriptome analysis results and *R. fascians* BLAST search results using Primer Premier TM 5.00. The expression studies were conducted following the RT-qPCR MIQE guidelines (Bustin et al. 2009), with three technical replicates for each of two biological replicates. The RT-qPCR assay was run using the KAPA SYBR® FAST qPCR Kits (Kapa Biosytems, Boston, USA). The temporal expression of each gene of interest and selected reference genes were quantified using a Qiagen Rotor-Gene Q.

To achieve accurate normalisation in the RT-qPCR, four reference genes *U18S, PsEF, PsGAP* and *PsACT*, were used as internal controls as described by Song et al. (2012) and Dhandapani et al. (2017). RT-qPCR was used to determine the relative expression of the genes of interest in pea shoots and roots at different growth stages following seed inoculation with the virulent strain (602), the avirulent strain (589) and the mock inoculated control. The expression data are presented as heat maps with fold differences calculated relative to the mock-inoculated control.

Cytokinin analyses

Pea shoots and roots from four individual plants from each of the three treatments, were ground under liquid nitrogen and freeze-dried. The four biological replicates were extracted and purified using the method described in Antoniadi et al. (2015), and Dhandapani et al. (2017). The samples were analysed by LC–MS/MS (Svačinová et al. 2012).

Results

Morphological and microscopic differences in the shoot and root of pea following inoculation with avirulent and virulent strains of *Rhodococcus fascians*

The morphological variations in the pea plants inoculated with the avirulent strain of *R. fascians* (avir-plants) and with the virulent strain of *R. fascians* (vir-plants) and mock inoculated (con-plants) are described in detail in Dhandapani et al. (2017). The characteristic multiple shoot symptoms were visible from 5 dpi in vir-plants and other symptoms such as reduced leaf, stunted shoot growth, shortened and thickened roots were evident from 11 to 45 dpi in vir-plants. Both avir-plants and con-plants had similar morphological characteristics throughout their growth period.

The chlorophyll content of vir-roots was low but increased with time, while the chlorophyll content of the vir-shoots was less relative to control and avir-shoots from 11 to 20 dpi and was only greater by 35 dpi (Fig. 1). The total chlorophyll content in vir-roots was greater than control and avir-roots especially at the later developmental stages (Fig. 1).

The multiple shoots emerging at 5 dpi were colonised by the virulent strain (Fig. 2a: C); by 15 dpi there was noticeable bacterial colonisation on the surface of the multiple shoots and on the single shoot of avir-plants (Fig. 2a: F). By 35 dpi, both strains of *R. fascians* appeared to colonise the shoot tissue in a similar manner (Fig. 2a: H, I). Through scanning electron microscopy, it was noticed that the colonisation of both avirulent and virulent strains of *R. fascians* on the surface of the pea tissues (root and shoot) increased with time from 5 to 35 dpi (Dhandapani 2014).

Both strains of *R. fascians* (avirulent and virulent) were detected on the radicle tissue of the pea at 2 dpi (Fig. 2b: B, C). The presence of mucilage-like material was seen near the surface of radicle root hairs of the roots infected by the avirulent strain (Fig. 2b: E) and the bacteria were mainly clumped together (Fig. 2b: B, E, H). The radicle infected by the virulent strain exhibited a layer of bacteria on the root epidermis and amongst the root hairs (Fig. 2b: C, F, I) which appeared as bacterial rods on enlargement (Fig. 2b: L). By 5 dpi, clumps of avirulent bacteria were evident on the root epidermis and root hairs (Fig. 2b: E), whereas the virulent bacteria were spread across the epidermal surface of the root amongst the root hairs (Fig. 2b: F). By 15 dpi, profuse colonies of virulent and avirulent bacteria were evident on the surface of the root (Fig. 2b: H, I). The avir-roots showed small rods clumped together on the root epidermis (Fig. 2b: E, H), whereas the virulent strain exhibited more diffuse spread of bacteria on roots (Fig. 2b: I, L).

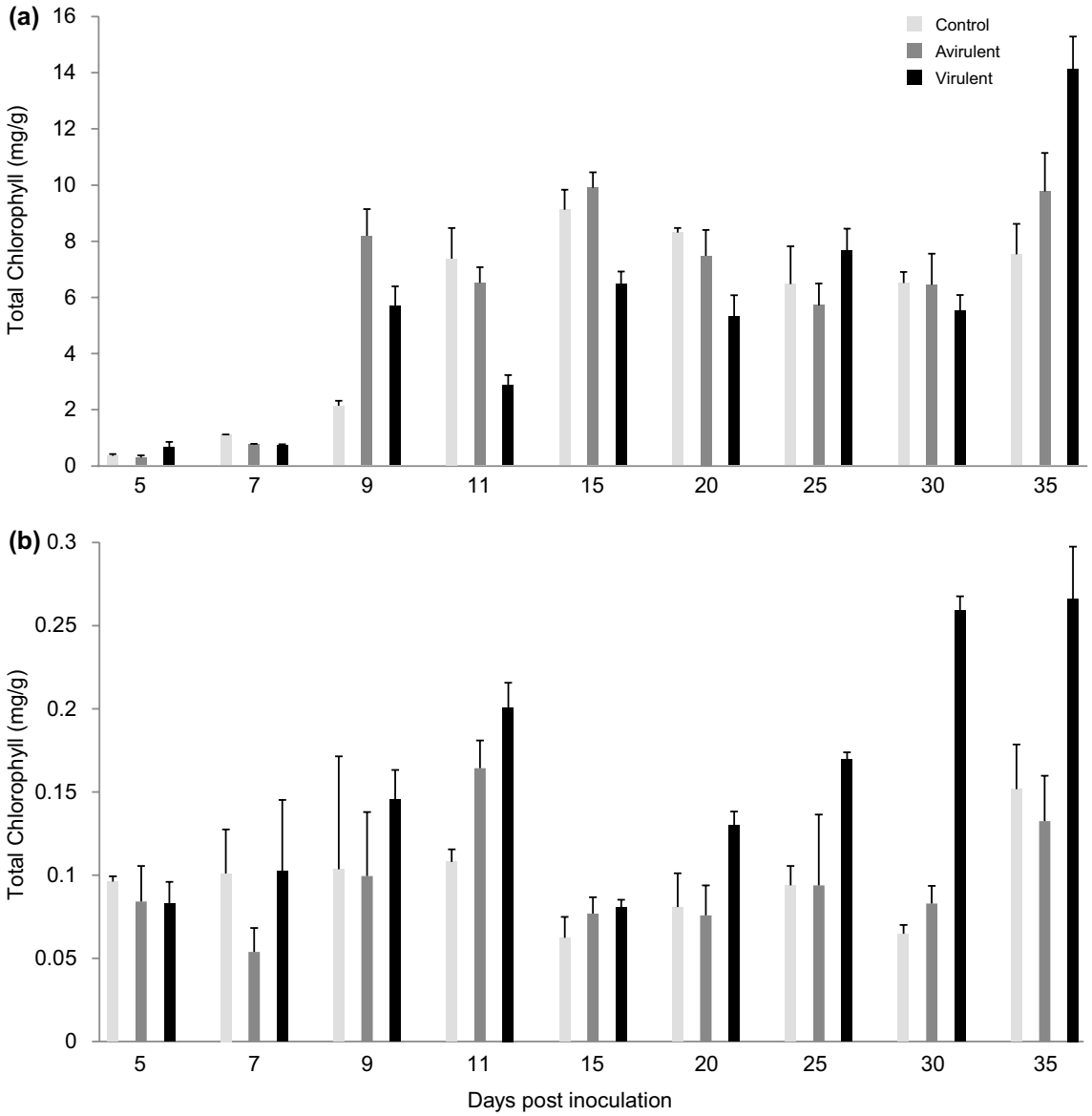

Fig. 1 Total chlorophyll content in **a** shoot and **b** root tissues of *P. sativum* infected with *R. fascians*. The pea seeds were imbibed for 4 h (hpi) with *R. fascians* avirulent strain 589 (avirulent), virulent strain 602 (virulent) and medium as mock inoculation (control) and grown in sterile agar containers until 35 days post inoculation (dpi). The error bars are ± SE of two biological replicates and four technical replicates

Expression of *RfIPT, RfCKX*, and *RfLOG* during infection of shoots and roots

In peas inoculated with the virulent strain of *R. fascians, RfIPT, RfLOG* and *RfCKX* showed markedly increased expression in shoots and roots by 15 dpi, and more so in roots (Fig. 3). No expression of *RfIPT, RfLOG* and *RfCKX* in control (con-shoots and con-roots) and shoots and roots inoculated with the avirulent strain (avir-shoots and avir-roots) was detected, confirming that the designed primers discriminated between the *R. fascians* genes and the pea genes as shown in Dhandapani et al. (2017).

Cytokinin biosynthesis and metabolism in shoot and root of pea following inoculation with avirulent and virulent strains of *Rhodococcus fascians*

In general, the expression of the cytokinin biosynthetic (*PsIPT*) and activation (*PsLOG*) gene family members in both vir- and avir-roots and shoots at 5 dpi was reduced relative to the controls (Figs. 4, 5). Expression of all *PsIPT* and *LOG* gene family members was elevated relative to controls from 15 to 35 dpi in vir-shoots and at 15 dpi in vir-roots. In the latter stages *PsIPT* and *LOG* expression was elevated in avir-shoots but not in avir-roots.

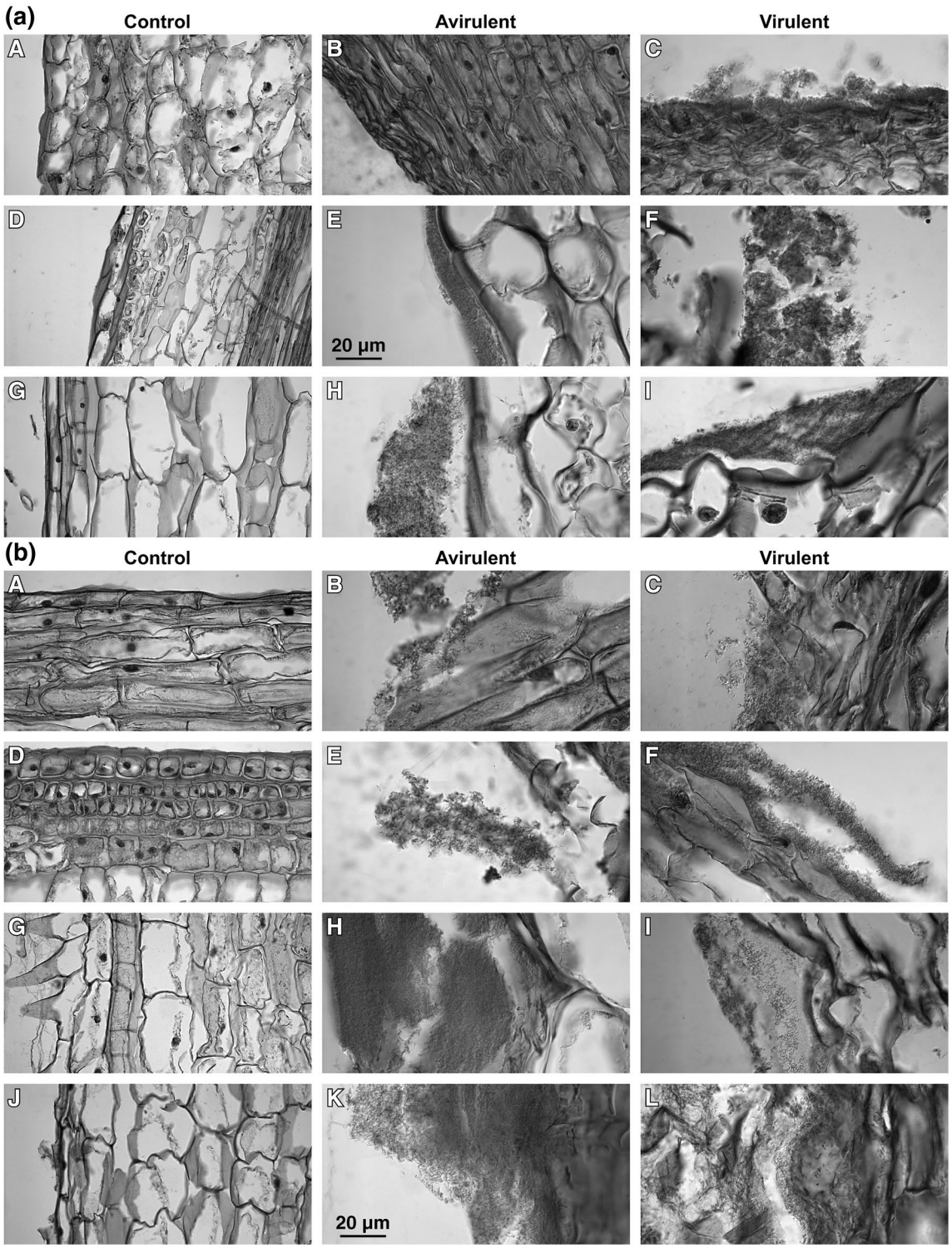

Fig. 2 Light micrographs of sections of *P. sativum* shoot and root following seed inoculation with *R. fascians*. **a** Light micrographs of sections of *P. sativum* shoot tissues following inoculation with *R. fascians* at 5 days post inoculation (dpi) (**A**–**C**), 15 dpi (**D**–**F**) and 35 dpi (**G**–**I**). **b** Light micrographs of sections of *P. sativum* root tissues following inoculation with *R. fascians* at 2 days post inoculation (dpi) (**A**–**C**), 5 dpi (**D**–**F**), 15 dpi (**G**–**I**) and 35 dpi (**J**–**L**)

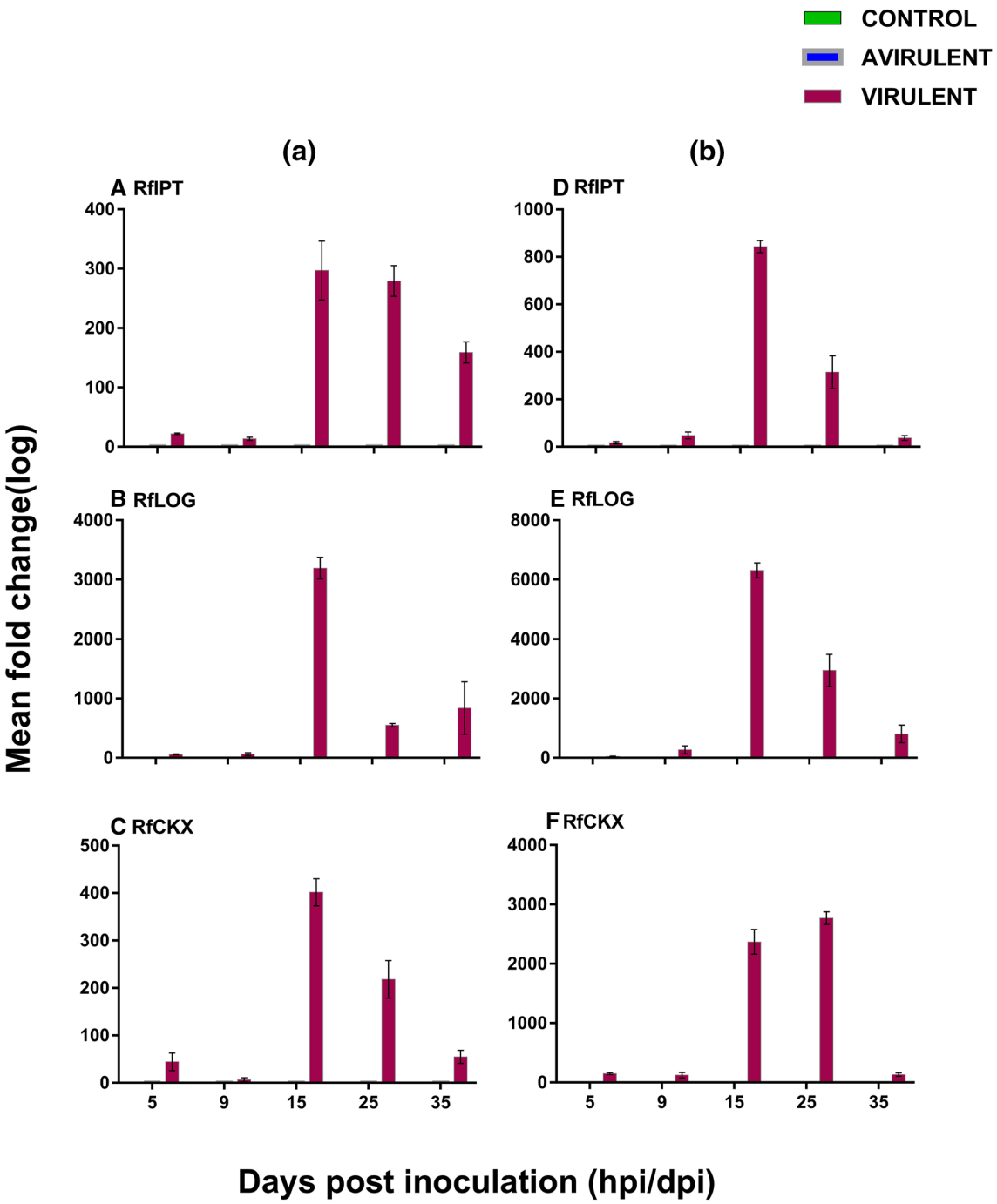

Fig. 3 Relative expression of *RfIPT, RfLOG* and *RfCKX* from *P. sativum* shoots and roots following seed inoculation with *R. fascians* strain 589 (avirulent), strain 602 (virulent) and mock inoculation (control) from 5 to 35 dpi. **a** Shoot and **b** root. Data are means of relative mRNA levels in fold changes (log) detected using RT-qPCR with three technical replicates for each of two biological replicates. *PsEF, U18S, PsGAP* and *PsACT* were used as internal controls. Error bars represent ±SD calculated for the combined technical and biological replicates

Expression patterns of *PsCKX* generally mirrored those of *PsIPT* and *LOG* (Figs. 4, 5). The expression of the *PsCKX* family members in both vir- and avir-shoots was elevated from 9 to 35 dpi (Fig. 4). *PsCKX2* expression was elevated at all time points, especially in vir-shoots. Expression of the *PsCKX* gene family members was increased in vir-and avir-roots relative to control, particularly at 15 dpi.

Expression of the cytokinin response regulator (*PsRR*) gene family members in both vir- and avir-shoots was generally elevated across all stages of development relative to

SHOOT

Target Genes	5 dpi VIR	9 dpi VIR	15 dpi VIR	25 dpi VIR	35 dpi VIR	5 dpi AVIR	9 dpi AVIR	15 dpi AVIR	25 dpi AVIR	35 dpi AVIR
PsIPT1	-16.8	-1.8	4.3	8.0	1.8	-7.8	3.1	-1.3	5.7	1.6
PsIPT2	-28.4	-1.0	8.5	7.3	3.2	-9.0	-6.1	1.4	10.7	2.4
PsIPT4	-12.3	1.2	2.9	7.1	5.0	-8.3	3.2	-1.7	2.2	3.1
PsLOG1	-10.4	1.6	15.3	5.4	3.4	-2.6	1.3	4.2	8.9	3.3
PsLOG6	-9.7	2.1	6.4	2.4	7.8	-1.4	7.9	2.3	4.4	6.0
PsLOG8	-4.2	3.0	46.7	1.3	2.0	1.0	2.9	2.5	4.7	4.5
PsCKX1	-2.2	5.1	5.4	8.5	6.6	-1.1	4.9	2.5	8.7	2.9
PsCKX2	3.8	31.8	32.3	66.6	6.0	6.4	9.6	2.2	15.0	2.7
PsCKX3	-11.5	3.6	8.8	15.5	2.6	-6.0	4.0	1.1	9.3	1.7
PsCKX5	3.6	4.3	10.3	20.3	7.9	5.9	2.7	2.3	9.3	4.0
PsCKX7	-1.3	2.1	1.7	5.3	7.0	1.6	1.9	2.1	8.5	3.6
PsRR3	1.8	20.9	13.1	46.8	9.7	2.1	12.6	1.2	9.2	4.8
PsRR5	3.0	10.9	120.3	15.2	5.6	6.8	2.4	2.1	3.8	2.2
PsRR6	7.3	2.8	6.0	15.7	2.4	25.9	6.7	2.6	3.0	4.2
PsRR9	23.5	12.2	19.6	211.9	2.9	19.3	10.0	1.8	27.0	1.4
PsAAP7a(Cluster 1)	-1.3	1.7	4.4	1.6	1.4	4.3	6.9	-2.1	6.4	2.0
PsAAP7b	1.9	17311.6	16.3	4.6	3.4	-1.4	3.8	37477.0	6.3	6.5
PsAAP2b(Cluster 3A)	10.0	3.7	2.8	1.8	3.1	19.6	10.3	-3.0	4.0	1.5
PsAAP2c	-2.9	-13.6	1.3	4.3	6.3	10.1	4.1	1.3	4.3	6.3
PsAAP3a	-22.1	14.2	2.8	3.5	1.7	-8.8	130.0	-1.0	2.5	1.6
PsAAP1(Cluster 4B)	2.0	3.6	8.6	4.5	3.0	2.8	11.1	-1.9	4.3	1.9
PsAAP6a	-14.0	-1.6	23.7	5.2	2.0	-2.1	-2.0	2.1	293.2	2.5
PsAAP8	-64.9	-2.2	19.3	9.8	5.8	-14.8	3.6	-3.0	30.0	6.0
PsSUT1	-2.9	3.0	2.3	2.5	1.7	-2.2	19.1	1.2	5.9	3.7
PsSUT2	-1.3	1.1	17.3	2.3	1.5	-1.6	1.1	2.0	2.5	-1.7
PsSUT5	-9.8	-1.8	10.2	17.4	8.1	-9.2	10.6	1.0	18.2	2.6
PsSUT3	1.2	-1.3	9.8	1.6	3.4	4.0	6.5	1.4	1.2	1.1
PsCWINV1	-129.8	-1.6	673.0	5.8	15.8	-77.7	5.1	3.2	10.0	7.7
PsCWINV2	2.0	1.4	7.1	13.6	1.5	7.7	6.8	-1.3	6.0	2.8
PsCWINV3	5.5	-3.2	8.3	2.0	1.9	34.8	4.3	-1.5	19.1	2.9
PsCWINV6	1.9	1.2	5.7	1.7	1.7	2.7	5.8	1.4	4.0	3.5
PsSW1(Clade I)	-4.6	-3.1	5.6	-1.2	-1.8	-1.4	7.4	1.7	3.3	-1.6
PsSW2a	6.3	1.0	10.7	2.8	2.0	20.4	6.9	3.7	3.8	2.3
PsSW2b	-15.6	-3.3	8.3	1.2	-4.9	-3.4	4.3	1.3	1.9	1.1
PsSW4(Clade II)	-41.5	-5.6	18.8	34.9	1.1	-2.0	9.7	-1.3	19.7	1.1
PsSW5a	-29.0	-2.5	19.4	11.3	1.3	-6.2	8.4	1.1	10.3	3.3
PsSW5b	-7.4	-1.7	5.7	2.0	1.3	-2.8	2.9	-1.9	2.5	1.2
PsSW7	2.5	3.2	1101.2	-1.1	61.0	3.9	14.1	2.4	13.3	23.4
PsSW9(Clade III)	-1.0	1.9	16.5	4.1	-1.3	2.9	10.2	-1.9	10.5	-3.4
PsSW12	-1.2	-1.0	4.3	-1.4	-4.3	1.4	7.9	1.6	1.2	-1.0
PsSW13	8.7	-3.6	2.2	-1.6	2.7	21.0	1.3	-1.5	1.5	1.8
PsSW15a	4.7	-2.8	145.6	-4.0	20.2	9.1	1.1	-1.2	1.6	6.6
PsSW15b	-3.9	-3.5	157.6	18.0	19.5	-4.7	12.2	-6.2	396.6	18.1
PsSW15c	-2539.8	-557.1	3.7	7.3	1.4	-2595.0	-4.3	-13.0	17.1	9.8
PsSW15d	19.1	5.5	3.4	5.3	1.4	38.6	22.6	-1.8	9.6	9.1
PsSW17(Clade IV)	-7.1	-2.8	4.4	3.3	-1.4	-1.6	5.9	-1.1	3.3	-1.3

>2000	1999.9 to200	199.9 to 20	19.9 to 2	1.9 to -1.9	-2 to -19.9	-20 to -199.9	<-200.0	ND
upregulated						downregulated		

Fig. 4 Relative expression of cytokinin biosynthesis (*PsIPT*), activation (*PsLOG*), degradation (*PsCKX*) and response regulator (*PsRR*) gene family members along with transporter (*PsAAP, PsSUT, PsCWINV* and *PsSW*) gene family members in *P. sativum* shoot tissues following seed inoculation with *R. fascians* strain 589 (AVIR) and virulent strain 602 (VIR) from 5 to 35 days post-inoculation. Values are fold changes relative to the expression of the mock-inoculated control. Initial fold change values were calculated using *PsEF, Ps18S, PsGAP* and *PsACT* as internal controls using three technical replicates for each of two biological replicates in the RT-qPCR. The relative expression level of each gene was then compared with the expression in pea shoots inoculated with medium as the mock-inoculated control. The colour scale indicates upregulated expression (red scale), similar (white) and downregulated expression (blue scale) relative to the mock-inoculated control. (Color figure online)

ROOT

Target Genes	5 dpi VIR	9 dpi VIR	15 dpi VIR	25 dpi VIR	35 dpi VIR	5 dpi AVIR	9 dpi AVIR	15 dpi AVIR	25 dpi AVIR	35 dpi AVIR
PsIPT1	-14.0	1.3	6.8	-1.4	-6.4	-85.0	-1.4	-2.2	-1.1	-65.0
PsIPT2	-11.6	1.6	16.2	1.7	-1.7	-31.3	-1.0	4.7	1.2	-3.9
PsIPT4	-9.9	-1.2	24.5	2.0	-1.2	-33.3	-1.8	6.3	1.5	-4.4
PsLOG1	-4.2	-1.4	46.3	2.1	1.1	-29.8	-7.9	6.8	1.7	-2.2
PsLOG6	-3.0	3.3	19.5	-1.1	-1.0	-9.2	-1.4	-1.2	-3.3	-4.5
PsLOG8	-1.3	8.5	19.1	3.8	2.7	-4.3	-2.2	-1.1	-1.8	-4.3
PsCKX1	-9.5	-1.4	12.4	1.7	-1.6	-41.9	1.6	5.6	3.3	-4.4
PsCKX2	-8.2	4.8	33.1	5.5	1.6	-49.5	-3.7	2.7	7.1	-10.2
PsCKX3	-6.5	1.3	14.4	1.6	-2.1	-16.5	1.1	6.5	3.9	-3.7
PsCKX5	-2.7	1.8	54.8	4.9	2.2	-87.9	-4.0	6.4	10.3	-10.9
PsCKX7	-2.2	-1.7	24.5	-1.2	-2.0	-72.7	-21.5	1.5	-3.0	-10.8
PsRR3	1.3	3.3	10.1	2.5	-2.2	-3.1	1.4	1.2	-1.7	-6.3
PsRR5	1.7	6.3	16.8	4.2	8.7	-12.2	-2.0	-3.3	-8.3	-2.7
PsRR6	-2.8	-2.6	15.4	1.7	-1.0	-20.9	-11.9	-3.9	-2.2	-5.9
PsRR9	-1.2	2.7	13.9	1.8	-2.3	-10.1	-11.2	-1.3	-1.9	-11.1
PsAAP7a(Cluster 1)	-8.8	1.2	21.6	1.3	2.0	-20.5	-4.0	5.9	-7.6	-1.2
PsAAP7b	-5.3	-1.6	21.3	-3.0	-1.6	-48.5	-1.6	9.1	-4.8	-1.9
PsAAP2b(Cluster 3A)	-1660.9	5.0	21.1	1.3	-7.6	-9.5	1.7	18.3	1.2	-3.7
PsAAP2c	-9.2	-1.8	11.8	2.2	-1.7	-18.3	-4.2	4.2	-3.7	-1.8
PsAAP3a	-65.3	-3.8	54.0	1.4	-15.3	-83.7	-12.9	32.3	2.2	-13.2
PsAAP1(Cluster 4B)	1.1	8.4	152.3	8.2	-2.4	-8.4	1.4	9.6	8.8	-71.1
PsAAP6a	-23.5	-3.3	790.1	10.9	1.0	-143.7	-46.5	110.7	1.4	-1.6
PsAAP8	-94.7	-65.1	40.6	1.2	-3.8	-357.4	-9.3	27.2	-1.0	-5.1
PsSUT1	-1.9	3.5	9.1	-2.4	-2.5	-9.8	7.0	8.3	2.7	-1.3
PsSUT2	-2.3	1.3	17.8	-1.2	-2.6	-8.6	-2.4	8.1	1.3	-7.8
PsSUT5	-4.5	1.9	273.0	2.3	-1.5	-42.4	-2.1	47.2	1.5	-2.3
PsSUT3	1.1	1.1	23.3	1.1	1.6	-3.9	1.6	9.0	-1.2	-3.1
PsCWINV1	1.1	-7.5	18593.1	12.2	14.2	-223.5	-3.9	430.9	1.9	-3.5
PsCWINV2	-51.6	-1.3	2.1	-7.4	-10.3	-9.2	-2.2	-1.8	-44.8	-1.0
PsCWINV3	-2.1	-7.5	329.5	52.5	2.0	-63.2	-105.5	93.3	49.6	-1.2
PsCWINV6	-21.4	-1.0	16.6	-2.5	-3.6	-11.1	-1.1	17.8	-3.3	-1.0
PsSW1(Clade I)	-6.2	-18.0	-1.8	-1.7	-73.0	-107.3	-37.3	1.7	-1.1	1.4
PsSW2a	-2.8	1.4	21.0	1.9	3.1	-10.6	-3.2	14.1	-3.7	1.4
PsSW2b	-8.1	-1.4	6.7	-3.7	-6.3	28225.4	-2.5	10.0	-10.1	-2.1
PsSW4(Clade II)	-19.2	-2.0	1331.1	7.6	1.2	-213.6	-4.9	95.7	6.5	-2.2
PsSW5a	-33.3	-4.1	173.1	2.3	-2.2	-155.4	-6.6	37.6	4.0	-3.5
PsSW5b	-1.6	-1.2	22.6	3.5	2.4	-12.9	-1.3	4.0	2.6	-1.2
PsSW7	20.0	41.0	2742.6	35.7	28.1	-15.7	1.5	230.8	1.1	5.7
PsSW9(Clade III)	-2.8	-9.7	11.1	3.1	25.1	-67.5	-288.9	34.9	-3.4	84.8
PsSW12	-35.7	-3.8	2.4	-1.6	-4.6	-27.0	-18.5	13.3	-2.6	3.4
PsSW13	-2.6	-29.0	3.6	1.3	-9.8	-4.0	-371.9	14.3	1.8	-2.2
PsSW15a	-9.9	-1.2	48.5	4.7	-1.6	-68.8	-1.3	6.9	-5.9	-1.6
PsSW15b	8.6	354.4	197828.8	416.8	363.0	-15.2	12.2	2656.1	6.4	65.7
PsSW15c	-431.9	-4.7	4.3	-8.0	-4.6	-60.8	1.9	5.1	-3.7	2.0
PsSW15d	-6.1	7.5	20.8	-1.1	-17.3	-27.7	3.7	14.4	-1.8	-5.9
PsSW17(Clade IV)	-2.4	2.4	6.7	-6.9	-5.4	-2.6	-2.9	4.9	-52.3	-5.9

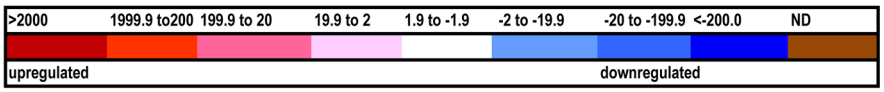

Fig. 5 Relative expression of cytokinin biosynthesis (*PsIPT*), activation (*PsLOG*), degradation (*PsCKX*) and response regulator (*PsRR*) gene family members along with transporter (*PsAAP, PsSUT, PsCWINV* and *PsSW*) gene family members in *P. sativum* root tissues following seed inoculation with *R. fascians* strain 589 (AVIR) and virulent strain 602 (VIR) from 5 to 35 days post-inoculation. Values are fold changes relative to the expression of the mock-inoculated control. Initial fold change values were calculated using *PsEF, Ps18S, PsGAP* and *PsACT* as internal controls using three technical replicates for each of two biological replicates in the RT-qPCR. The relative expression level of each gene was then compared with the expression in pea shoots inoculated with medium as the mock-inoculated control. The colour scale indicates upregulated expression (red scale), similar (white) and downregulated expression (blue scale) relative to the mock-inoculated control. (Color figure online)

control (Fig. 4). However, in vir-roots expression of the *PsRRs* was suppressed at 5 dpi but increased at 9 and 15 dpi. In contrast, in avir-roots *PsRR* expression was frequently less than the relevant control.

The total cytokinin content of the vir-shoots was reduced significantly relative to control and avir-shoots at 5 and 11 dpi, but then increased at 15 and 25 dpi relative to con-shoots. At these latter time points the cytokinin content was similar in avir- and vir-shoots (Table 1). The total cytokinin content of avir-roots was significantly greater than controls at 5 and 15 dpi, whereas that in vir-roots was significantly less at 11 dpi, but greater at 15 dpi. Cytokinin levels were similar in avir and vir-roots at 15 and 25 dpi (Table 1).

Considering the individual cytokinins, there was significantly less tZ, tZRMP, iPR and iPRMP in vir-shoots 5 and 11 dpi relative to control. iP levels were consistently elevated relative to controls and avir-shoots. However, the total iP-type cytokinins were less in the vir-shoots relative to controls and avir-shoots at 5 and 11 dpi, but greater at 15 and 25 dpi.

Initially, there was no difference in the total iP-type cytokinins in the roots at 5 dpi. Subsequently, the iP-type cytokinins in the vir-roots only exceeded those in the controls at 25 dpi. The iP levels were elevated relative to control at the four time points in vir-roots. However, at both 11 and 15 dpi the iP levels in both vir- and avir-roots were significantly elevated relative to control. At 5 dpi, tZ-type cytokinins were elevated in vir-roots relative to con-roots, particularly tZR. However, at 11 dpi most cytokinin forms, including tZ, were decreased in vir-roots, while at 15 and 25 dpi free bases and nucleotides were generally elevated.

Transporter gene expression in *Rhodococcus fascians*-inoculated pea tissues

The expression of sucrose transporter (*PsSUT*) gene family members was similar in both vir- and avir-shoots at 5 dpi and was reduced relative to con-shoots. Subsequently, expression was increased or was similar to controls in both vir-and avir-shoots (Fig. 4). Likewise, the expression of the *SUT* gene family members was similar in both vir- and avir-roots: decreased expression at 5 and 9 dpi and elevated expression particularly at 15 dpi (Fig. 5).

Generally, the *PsAAP* gene family members showed decreased expression relative to controls at 5 dpi. At 15 dpi expression relative to controls was greater in the vir-shoots than the avir-shoots. Cluster 1 *PsAAP 7a* expression was high at 9 dpi in vir-shoots and at 15 dpi in avir-shoots (Fig. 4). The relative expression of Cluster 4B *AAP6a* and *8* was greater at 15 dpi in vir-shoots and at 25 dpi in avir-shoots. In general, for many *PsAAP* gene family members, the expression in vir-and avir-roots was reduced relative to

Table 1 Endogenous cytokinin content in *Pisum sativum* shoot and root following seed inoculation with *Rhodococcus fascians*

Cytokinin (pmol/g DW)	Time points											
	5 dpi[a]			11 dpi			15 dpi			25 dpi		
	CON	AVIR	VIR	CON	AVIR	VIR	CON	AVIR	VIR	CON	AVIR	VIR
Shoot												
Total CK	357.41	397.43	191.2***	93.97	112.86	53.25***	55.83	77.95**	78.69***	43.68	51.63**	64.6***
tZ	7.16	6.58	5.01**	3.81	4.17	0.85***	1.74	2.34	1.53	1.74	1.86	1.76
tZR	4.01	2.96*	3.59	1.86	1.02**	0.1***	0.47	0.29**	0.34*	0.28	0.45*	0.8***
tZRMP	23.87	35.65**	18.37**	11.14	8.73	0.93***	2.19	1.53*	2.19	0.61	1.28***	2.21***
iP	7.34	6.87	24.62***	2.79	3.32	7.6***	2.63	3.39	17.69***	2.41	2.28	11.01
iPR	13.12	11.11	6.34***	2.88	2.84	1.12**	2.98	1.53**	2.02*	1.33	0.91*	2.02**
iPRMP	142.1	136.52	48.83***	22.66	20.83	9.37***	10.76	18.41***	22.8**	6.91	7.42	11.98**
Root												
Total CK	197.9	277.92*	216.53	220.5	155.6**	75.05***	113.68	171.92***	163.53***	94.11	93.96	105.4*
tZ	4.55	4.64	5.86**	5.75	4.29**	1.76***	3.83	4.98	5.14*	1.33	2.03*	1.72*
tZR	6.78	5.16*	16.62***	9.13	3.36***	1***	6.21	3.19**	5.32	0.75	1.05*	2.11***
tZRMP	15.17	32.12**	28.69**	37.66	13.81***	6.8***	6.68	12.82**	29.47***	1.27	1.5	5.23***
iP	5.82	7.32	14.02***	3.62	8.17**	6.88***	3.53	5.74***	8.69***	3.22	3.02	7.35***
iPR	19.19	16.61	14.37*	11.24	9.29	2.75***	24.44	11.24**	7.87***	7.81	4.84**	8.73
iPRMP	69	96.8	57.59	68.53	42.15*	15.51***	16.32	42.7***	34.68**	10.09	7.89	23.95***

Data for the control have been published previously in Jameson et al. (2016). The data are the averages of four biological replicates

dpi days post-inoculation, *CON* mock-inoculated control, *AVIR* seeds were inoculated with the avirulent strain, *VIR* seeds were inoculated with the virulent strain

*, **, ***Value is significantly different from the mock-inoculated control $p \leq 0.05$; $p \leq 0.01$; $p \leq 0.001$, respectively

control at 5, 9 and 35 dpi but elevated at 15 dpi and more so in the vir-roots than the avir-roots (Fig. 5).

Generally, expression of the *PsCWINVs* was only modestly increased in both vir- and avir-shoots relative to the controls, with the exception of *PsCWINV1* at 15 dpi in vir-shoots (Fig. 4). Expression in vir-roots was generally decreased relative to controls, with the marked exception at 15 dpi where expression was elevated for the four family members, and more strongly in the vir-roots than the avir-roots (Fig. 5).

Expression of Clades I, II and III SWEET gene family members was usually decreased in vir-shoots at 5 and 9 dpi, but increased at 15 dpi (Fig. 4). *SWEET* expression in the avir-shoots was more variable and lacked the increase at 15 dpi seen in the vir-shoots. Clade III *SW15c* was markedly decreased in both vir- and avir-shoots. In vir-roots, *SWEETs* were noticeably elevated relative to controls at 15 dpi, more so in the vir-roots than the avir-roots (Fig. 5). Clade II *PsSW7* was strongly elevated relative to controls across the experiment in vir-roots, as was Clade III *SW15b* in both avir- and vir-roots. In contrast, expression of most *SWEETS* in vir-and avir-roots was decreased relative to controls at 5 and 9 dpi.

Discussion

Rhodococcus fascians is a soil bacterium. We were interested in the effect of seed inoculation, as most previous research has focused on infection of above ground tissues. The virulent strain of *R. fascians* transformed the cotyledon from being a sole source for the germinating seed to a competitive source of nutrients for the pathogen (Dhandapani et al. 2017). As the integrity of the cotyledon was maintained by the virulent strain, we investigated whether there was an ongoing impact from the presence of the bacterium on the emerging roots and shoots of seeds that were inoculated with either the virulent or the avirulent strain.

We previously showed that there was a noticeably greater impact of the virulent strain on gene expression in the cotyledons compared to the avirulent strain, especially between 2 and 15 dpi (Dhandapani et al. 2017). However, as the shoot developed, on first examination of the heat map (Fig. 4) both strains led to increased expression of the genes of interest, but the differences in expression between the virulent and avirulent strains are not particularly pronounced, even though morphologically the inoculated plants were extremely different. This supports the notion of the avirulent epiphytic strain of *R. fascians* also affecting the metabolism of the plant (Depuydt et al. 2009; Dhandapani et al. 2017). Whether this is due to cytokinin production by the bacteria is not known, but both virulent and avirulent strains of *R. fascians* produce substantial quantities of non-hydroxylated cytokinins in vitro (Eason et al. 1996) as well as methylthio derivatives (Francis et al. 2016)—potentially from the turnover of tRNA (Matsubara et al. 1968; Jameson 2000).

However, at 5 dpi, when symptoms are first becoming apparent, there is reduced expression of *PsIPT* and *PsLOG* in vir-shoots compared with avir-shoots, relative to controls. This matches the reduced levels of the Z-type and iP-type endogenous cytokinins in the vir-shoots relative to controls and avir-shoots, indicating that the pathogen continues to impact the homeostatic mechanisms of the germinating plant. Expression of *PsCKX* gene family members is apparent in both the vir- and avir-shoots, but with noticeably greater *PsCKX2* expression occurring in vir-shoots. *PsCKX* expression is particularly evident in the vir-roots at 15 dpi, as is *PsIPT* and *LOG* expression. *CKX* expression and activity frequently parallels enhanced *IPT* expression and increased endogenous cytokinin levels (see references cited in Jameson and Song 2016), and the virulent strain (but not the avirulent strain) of *R. fascians* has been shown to specifically upregulate *AtCKX3* in transgenic tobacco (Gális et al. 2005b).

Increased expression of the *RR* gene family members is indicative of enhanced cytokinin levels and/or perception (Hwang et al. 2012). Increased *PsRR* expression relative to the controls in the inoculated shoots and the vir-roots indicates that they are responding to more cytokinin than the control shoots. The lack of *PsRR* gene expression is particularly noticeable in the avir-roots. A similar differential for *AtIPT, AtCKX* and *AtRR* response has been reported for arabidopsis leaves inoculated with either a virulent (D188) strain or its avirulent equivalent (D188-5) (Depuydt et al. 2008, 2009).

Detection by the plant of the bacteria is also evidenced in early shoot growth with down-regulation of several *SWEETS*, but particularly a Clade III *SWEET*. SWEETs in this clade move sucrose across the plasma membrane to the apoplast (Chen 2014). Down-regulation would remove a source of carbohydrate from the bacteria. However at 15 dpi, the expression of *PsCWINVs, PsSWEETS, PsSUTs* and *PsAAP* gene family members are all upregulated in the vir-shoots but not the avir-shoots. At this time the expression of the *R. fascians* genes was strongly detected in the shoots and roots (Figs. 4, 5) and bacterial growth was prolific (Fig. 2). Clearly, the virulent strain was having a marked impact on the plant shoot as a source of nutrients. However, subsequently, both epiphytic and endophytic strains continued to affect nutrient transporters in the shoot, as noted also by Depuydt et al. (2009). The ability of an epiphytic strain to utilise plant-derived carbon sources was recently reported by Francis et al. (2016).

The impact of both the virulent and avirulent strains on transporters in the roots was clearly evident by 15 dpi, again when expression of *RfIPT, LOG* and *CKX* was detected and when bacterial colony growth was profuse. However, while

the patterns of expression are remarkably similar, there is a stronger upregulation of gene expression in the vir-roots compared with the avir-roots at 15 dpi. Generally, there is a greater level of down-regulation of the gene families of interest by the avir-roots compared to the avir-shoots. The one exception is the noticeable up-regulation of *PsSW2b* in the avir-roots. SWEETS in Clade I are associated with the transport of hexoses across either the plasmalemma or the tonoplast (Chen 2014). Transport across the tonoplast would sequester the glucose away from the bacteria (Chen et al. 2015). We suggest that the plant has actively combated the demands of the epiphytic strain more successfully than the virulent strain, and more successfully in the roots than the shoots.

Cytokinin is strongly implicated in the development of chloroplasts and the accumulation of chlorophyll (Cortleven and Schmülling 2015), and in the transition of etioplasts to chloroplasts (Cortleven et al. 2016). Chlorophyll accumulated in the cotyledons of the peas infected by the virulent strain, and Dhandapani et al. (2017) suggested that this was an indicator of enhanced cytokinin levels in the infected cotyledons. Likewise, the vir-roots accumulated more chlorophyll than the avir-roots or controls, which again could be caused by enhanced cytokinin levels. Recently, Kobayashi et al. (2017) showed chloroplast development in arabidopsis roots occurred through cytokinin response regulator signalling. We show here that expression of *PsRRs* was elevated in vir-roots, supporting the suggestion that the vir-roots, but not the avir-roots, were responding to an elevated cytokinin content.

Root growth is inhibited by the virulent *R. fascians* (Eason et al. 1995; Dhandapani et al. 2017). As endogenous cytokinin levels in roots are considered to be supraoptimal (Werner et al. 2003), and applied cytokinin inhibits root growth (Guo et al. 2017), further inhibition could be expected if *R. fascians* was increasing endogenous cytokinin levels. The inference of greater cytokinin levels in the vir-roots is described above with respect to chlorophyll accumulation. However, in terms of the individual cytokinins measured, there is a lack of correlation between the cytokinin content of the vir-roots (growth inhibition) and the similar or greater cytokinin content of the avir-roots (no growth inhibition). Neither do the data support the contention of the iP-type cytokinins being responsible for the root inhibition caused by the virulent strain. As the methylated cytokinins are reported to inhibit root growth in arabidopsis (Radhika et al. 2015), we are currently investigating the effect of these cytokinins in pea.

In conclusion, both the virulent and avirulent strains influenced transporter gene expression in the developing root and shoot of inoculated pea plants, with a more generally positive manipulation of transporter expression in the shoots over time, but with a lesser effect on the roots until a significant mass of bacteria had accumulated. Both strains are capable of releasing cytokinin (Eason et al. 1996) and, while this may affect transporter gene expression, it appears that the 'classical' cytokinins, the iP- and Z-types, are unlikely to be the causative molecules of the extreme symptoms invoked by the virulent strain of *R. fascians*.

Acknowledgements A UC scholarship to PD is gratefully acknowledged. Thanks to Graeme Bull, Jan McKenzie and Neil Andrews for assistance with microscopy, and to the anonymous referees for their constructive comments. O.N. was funded by the Czech Science Foundation (Nr. 17-06613S).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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