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Research

Differing requirements for flavonoids during the formation of lateral roots, nodules and root knot nematode galls in *Medicago truncatula*

Anton P. Wasson[1], Kerry Ramsay[2], Michael G. K. Jones[2] and Ulrike Mathesius[1]

[1]Australian Research Council Centre of Excellence for Integrative Legume Research, Department of Biochemistry and Molecular Biology, School of Biology, Australian National University, Canberra ACT 0200, Australia; [2]School of Biological Sciences and Biotechnology, Murdoch University, South Street, Murdoch, Western Australia 6150, Australia

Author for correspondence:
Ulrike Mathesius
Tel: +61 2 6125 2840
Email: Ulrike.Mathesius@anu.edu.au

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Summary

• In this study, we tested whether the organogenesis of symbiotic root nodules, lateral roots and root galls induced by parasitic root knot nematodes (*Meloidogyne javanica*) was regulated by the presence of flavonoids in the roots of *Medicago truncatula*. Flavonoids accumulate in all three types of root organ, and have been hypothesized previously to be required for secondary root organogenesis because of their potential role as auxin transport regulators.

• Using RNA interference to silence the flavonoid biosynthetic pathway in *M. truncatula*, we generated transformed flavonoid-deficient hairy roots which were used to study flavonoid accumulation, cell division and organogenesis of nodules, lateral roots and root galls.

• Flavonoid-deficient roots did not form nodules, as demonstrated previously, but showed altered root growth in response to rhizobia. By contrast, flavonoid-deficient roots showed no difference in the number of lateral roots and root galls. Galls on flavonoid-deficient roots formed normal giant cells, but were shorter, and were characterized by reduced numbers of dividing pericycle cells.

• We rejected the hypothesis that flavonoids are required as general regulators of the organogenesis of secondary root organs, but flavonoids appear to be necessary for nodulation. Possible reasons for this difference in the requirement for flavonoids are discussed.

Abbreviations: *CHS*, chalcone synthase gene; DPBA, diphenylboric acid-2-aminoethyl ester; GFP, green fluorescent protein; HPLC, high-performance liquid chromatography; J2, second-stage juvenile; RKN, root knot nematode; RNAi, RNA interference.

Introduction

The plant root system displays considerable plasticity in its growth and development. Although root growth occurs as a result of cell divisions in the root apical meristem, secondary root organs can be formed as a result of divisions in already differentiated cell types, including the pericycle, cortex and vascular parenchyma of the root. These secondary root organs include lateral roots, which develop on all land plants; nodules, which are formed in symbiosis with nitrogen-fixing bacteria on legumes and actinorhizal plants; and root galls, which can develop on many plant hosts infected with plant parasitic nematodes. One question that has been raised is whether these different organogenesis programmes involve common regulatory mechanisms (Hirsch & LaRue, 1997; Koltai *et al.*, 2001; Gheysen & Fenoll, 2002; Mathesius, 2003; Bird, 2004; Davis & Mitchum, 2005).

Lateral roots are typically initiated from dividing pericycle cells opposite xylem poles at a certain distance from the root apical meristem (Dubrovsky *et al.*, 2000). Pericycle founder cells of lateral roots remain in a meristematic state after emerging from the root apical meristem (Beeckman *et al.*, 2001). Cyclic

pulses of increased auxin response in the root basal meristem are likely to specify lateral root initials (De Smet *et al.*, 2007).

Nodule organogenesis is triggered by specific signal molecules (Nod factors) produced by nitrogen-fixing bacteria, called rhizobia, which form specific symbioses with many legume species. Nodule organogenesis falls into two categories based on the legume host. Nodules originate in pericycle and inner cortical cells in legumes forming indeterminate nodules (e.g. *Medicago truncatula*), whereas determinate nodules originate from cell divisions and cell enlargement in the outer cortex (Hirsch, 1992).

Plant parasitic nematodes infect a wide range of host plants. Root knot nematodes (RKNs) (*Meloidogyne* spp.) trigger the formation of root galls characterized by the formation of giant cells (Jones, 1981). Giant cells are typically initiated in vascular parenchyma cells, which undergo repeated mitoses without cytokinesis, followed by endoreduplication (Christie, 1936; Jones & Payne, 1978; Goverse *et al.*, 2000a). The expansion of several giant cells in the vascular cylinder is usually accompanied by cell divisions in the surrounding pericycle and cortical cells (Christie, 1936; Goverse *et al.*, 2000a; Karczmarek *et al.*, 2004).

Although the organogenesis of lateral roots, nodules and galls is initiated by different triggers, it is thought to involve common aspects, in particular the regulation of cell division and differentiation by the plant hormones auxin and cytokinin (Hirsch *et al.*, 1997; Goverse *et al.*, 2000a,b; Lohar *et al.*, 2004; Gonzalez-Rizzo *et al.*, 2006; Curtis, 2007; Fukaki *et al.*, 2007; Mathesius, 2008). Auxin is necessary to stimulate the first divisions in the pericycle leading to lateral root development (Dubrovsky *et al.*, 2008), and studies with auxin-responsive reporter genes have shown high expression in the first dividing pericycle cells (Benkovà *et al.*, 2003). Similarly, high activity of auxin response genes has been demonstrated in the first dividing pericycle and inner cortical cells of indeterminate nodules (Mathesius *et al.*, 1998b; van Noorden *et al.*, 2007), in outer cortical cells of developing determinate nodules (Pacios-Bras *et al.*, 2003) and in pericycle, cortex and giant cell precursors of root galls (Hutangura *et al.*, 1999; Karczmarek *et al.*, 2004; Wang *et al.*, 2007). This suggests that auxin plays a similar role in the regulation of the early cell divisions in all three organogenesis systems. In addition, there is evidence that correct auxin transport regulation is crucial for lateral root initiation (Casimiro *et al.*, 2001; Bhalerao *et al.*, 2002) and indeterminate nodulation (Mathesius *et al.*, 1998b; Boot *et al.*, 1999). Auxin transport has not been measured during gall formation, but the expression of auxin-responsive reporters is reduced below an initiating gall (Hutangura *et al.*, 1999; Karczmarek *et al.*, 2004) and, during cyst nematode infection, PIN-mediated auxin transport changes are necessary for cyst formation (Grunewald *et al.*, 2009).

Certain plant metabolites are known to regulate auxin transport. Flavonoids are phenylpropanoids with activities as antioxidants, enzyme regulators, antimicrobial compounds, pigments and signals to microorganisms (Winkel-Shirley, 2001). Certain flavonoids regulate polar auxin transport (Jacobs & Rubery, 1988) and auxin breakdown (Furuya *et al.*, 1962; Mathesius, 2001). Therefore, it has been suggested that flavonoids could be developmental regulators that control organogenesis via their effects on auxin transport and localization (Taylor & Grotewold, 2005; Peer & Murphy, 2007), in particular during nodulation (Hirsch *et al.*, 1989; Mathesius *et al.*, 1998b) and root gall formation (Hutangura *et al.*, 1999; Mathesius, 2003).

Flavonoids accumulate specifically in dividing cells in the root, for example in the lateral root and nodule primordia of subterranean clover (*Trifolium subterraneum*) (Djordjevic *et al.*, 1997; Morris & Djordjevic, 2006), white clover (*Trifolium repens*), pea (*Pisum sativum*) and siratro (*Macroptilium atropurpureum*) (Mathesius *et al.*, 1998a), in the lateral root primordia of Arabidopsis (Peer *et al.*, 2001), in the developing galls of white clover (Hutangura *et al.*, 1999), and in the root tip of Arabidopsis (Peer *et al.*, 2001; Buer *et al.*, 2007). In animal cells, flavonoids have activities as kinase inhibitors, cell cycle regulators and mimics of oestrogen (Kuo, 1997; O'Prey *et al.*, 2003). Although the activity of flavonoids as direct regulators of cell cycle activity or gene expression has not been tested in detail in plants, it is likely that flavonoids could play such roles in plants, and this could explain their localization in dividing cells and in plant nuclei (Peer *et al.*, 2001; Polster *et al.*, 2006).

In support of a role for flavonoids as developmental regulators, Arabidopsis mutants with altered flavonoid biosynthesis show various developmental defects, ranging from lack of gravitropism (Buer *et al.*, 2006), root looping and aberrant root outgrowths (Buer & Djordjevic, 2009) to defects in whole plant growth (Besseau *et al.*, 2007). Altered lateral root density has also been observed in flavonoid-deficient mutants, although this is dependent on the growth conditions (Brown *et al.*, 2001; Buer & Djordjevic, 2009). In addition, treatment of embryogenic cell cultures with certain (iso)flavonoids inhibits root formation (Imin *et al.*, 2007). Lack of flavonoids in Arabidopsis mutants does not affect the reproduction of RKNs (Wuyts *et al.*, 2006a) or cyst nematodes (Jones *et al.*, 2007), although effects of flavonoids on gall development were not analysed. In legumes, silencing of the flavonoid pathway has only been made possible via RNA interference (RNAi) as most genes encoding enzymes of the flavonoid pathway occur as multigene families, in contrast with Arabidopsis. Silencing of the flavonoid synthesis pathway in barrel medic (*M. truncatula*) abolished nodule initiation, and this was accompanied by the inability of rhizobia to inhibit auxin transport (Wasson *et al.*, 2006). Recently, the targeted silencing of different branches of the flavonoid pathway in *M. truncatula* has shown that the flavonol kaempferol is most likely to mediate auxin transport inhibition during nodulation (Zhang *et al.*, 2009).

Although different studies have examined nodulation, lateral root formation and nematode infection in different

flavonoid-deficient plant species, as described above, the three developmental processes have not been studied in detail under comparable conditions in one plant species. Results for lateral root formation in flavonoid-deficient Arabidopsis plants have been contradictory, and gall development, other than nematode reproduction, has not been examined. To test the hypothesis that flavonoids are required for the correct organogenesis of lateral roots, nodules and galls, we used the model legume *M. truncatula* as a plant species forming all three types of organ. We generated hairy roots transformed with a previously designed RNAi construct targeted against the chalcone synthase gene (*CHS*), encoding the first enzyme of the flavonoid pathway (Wasson *et al.*, 2006), to characterize the formation of lateral roots, nodules formed in symbiosis with the symbiont *Sinorhizobium meliloti* and root galls caused by infection with the RKN *Meloidogyne javanica*.

Materials and Methods

Bacterial strains

The green fluorescent protein (GFP)-labelled *S. meliloti* strain Rm1021 (Gage *et al.*, 1996) was maintained on Bergensen's modified medium (Rolfe *et al.*, 1980) containing 50 μM tetracycline. The *Agrobacterium rhizogenes* ARqua1 strain (Boisson-Dernier *et al.*, 2001) was maintained on tryptone yeast medium containing 100 μg ml^{-1} streptomycin.

Generation of *CHS* RNAi vector and transformation of *A. rhizogenes*

Two different vectors were used to silence *CHS*. For fluorescence microscopy, we transformed plants with the RNAi vector used to silence *CHS* transcripts, as described in Wasson *et al.* (2006), which contains a hairpin construct against *CHS* in the pHellsgate8 RNAi vector. To improve screening for transformed roots in all other phenotypic assays, the pDONR201-CHSi vector, described in Wasson *et al.* (2006), was recombined into pK7GWIWG2D(II) (Karimi *et al.*, 2002) using Gateway cloning. This hairpin-producing RNAi vector contains a constitutively expressed eGFP element for improved screening in plant transformation. Recombination reactions were performed according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The inserts were verified by restriction digests and sequencing. The verified construct was then transformed into the *A. rhizogenes* strain ARqua1 using the freeze–thaw transformation method of Hofgen & Willmitzer (1988).

Hairy root transformation and plant growth conditions

Medicago truncatula Gaertn. cv. Jemalong A17 seeds were scarified on sandpaper, sterilized in 6% (w/v) sodium hypochlorite for 10 min and thoroughly rinsed in sterile water. Seeds were vernalized at 4°C overnight and germinated at 25°C in the dark. The transformation of *M. truncatula* with *A. rhizogenes* followed the technique of Boisson-Dernier *et al.* (2001). Transformation with empty pHellsgate8 or pK7GWIWG2D(II) vector was used throughout the study as a control. Composite plantlets were grown on 15-cm-diameter Petri dishes containing sloped modified Fåhraeus medium with kanamycin (25 mg l^{-1}) (Boisson-Dernier *et al.*, 2001). Plantlets were grown at 20°C for 7 d, before being transferred to 25°C. A 16-h light period with a light intensity of 150 μmol was used throughout. When hairy roots were *c.* 1 cm in length, they were screened for GFP fluorescence as a secondary marker for successful transformation. Untransformed roots were excised. Plants were then transferred to plates containing Fåhraeus medium without kanamycin.

Meloidogyne javanica culture

Tomato seeds (*Lycopersicum esculentum* cv. *Grosse Lisse*) were rinsed for 5 min in 70% ethanol, for 20 min in 4% (w/v) sodium hypochlorite and washed with sterile water. Seeds were germinated and grown horizontally on Murashige and Skoog (MS) phytagel plates (Sigma, St Louis, MO, USA) at 25°C, with a day : night cycle of 16 h : 8 h at *c.* 100 μmol for 2 wk. Egg sacks of *Me. javanica* were collected from previously infected plants and resuspended in a droplet of sterile distilled water on a Petri dish and incubated for 48 h to hatch. Hatched juvenile *Me. javanica* were then inoculated onto the exposed tomato roots. Cultures were maintained by regularly reinfecting fresh tomato seedlings with newly hatched infective second-stage juveniles (J2s). When used experimentally, the J2s were inoculated onto hairy roots of *M. truncatula* of *c.* 1.5–2 cm in length. To ensure that similar numbers of J2s were used to inoculate each root, an aliquot of the same suspension of J2s in water was used for each inoculation. After counting the number of J2s in several aliquots under a stereomicroscope, the suspension was adjusted to contain *c.* 20 J2s for every root inoculation. In experiments on untransformed seedlings, this resulted in more than 90% of wild-type roots becoming infected, although we observed generally lower rates of infection in hairy roots.

Microscopy

All microscopy was carried out with roots transformed with the pHellsgate8 vector to avoid GFP fluorescence interfering with flavonoid fluorescence under UV light. Roots were cut into 5-mm segments and immediately embedded in 3% (w/v) agarose. They were then cross-sectioned at 150 μm thickness on a vibratome (1000 plus, Vibratome Company, St Louis, MO, USA) and viewed immediately under an epifluorescence microscope (Leica DMLB, Wetzlar, Germany). Flavonoids were stained by incubating fresh sections for 5 min in diphenylboric acid-2-aminoethyl ester (DPBA; Sigma) (0.5% in 10 mM

phosphate buffer, 2% dimethylsulphoxide, 1% sucrose, pH 6) and viewing sections under a UV excitation filter (excitation maximum at 365 nm, 425 nm longpass filter). Toluidine blue staining was performed by incubating sections for 5 min in 0.5% toluidine blue (pH 4.4 in sodium benzoate buffer), washing sections with distilled water and viewing under bright field illumination. Images were taken with a mounted CCD camera (RT Slider, Diagnostic Instruments, Sterling Heights, MI, USA). Exposure settings for fluorescent images were kept constant.

High-performance liquid chromatography (HPLC)

Roots of empty vector control or *CHSi* hairy root plantlets, both transformed with the pK7GWIWG2D(II) vector, were screened for GFP to select transformed roots. Extraction and separation of flavonoids were carried out in a similar manner to Farag *et al.* (2007) as follows. Root material was immediately ground in liquid nitrogen, 1 ml of 80% methanol was added per 100 mg fresh weight and the sample was extracted for 16 h at 22°C in the dark on a rotating wheel. Tubes were centrifuged at 14 000 rpm on a bench centrifuge at room temperature for 30 min. The supernatant was evaporated in a Speedvac centrifuge. The residue was redissolved in 2 N HCl and heated at 80°C for 90 min to deglycosylate flavonoids. The acid hydrolysate was mixed with two volumes of ethyl acetate, shaken and the ethyl acetate fraction was dried in a Speedvac centrifuge. The residue was redissolved in 45% methanol at 1 ml g^{-1} original fresh weight. Flavonoids were separated on a Shimadzu LC-10 VP series high-performance liquid chromatograph (Shimadzu Australasia, Sydney, New South Wales, Australia), equipped with a diode array UV–VIS detector and a fluorescence detector, using an Alltec Altima C18 5 μm reverse phase column (250 × 4 mm; Alltech Associates, Baukham Hills, Australia). The solvents were as follows: A, 0.1% aqueous acetic acid; B, acetonitrile + 0.1% acetic acid. Separation was over 70 min as follows: 0–5 min, 100% A; 5–70 min, 0–80% B using a linear gradient. Absorbance was recorded between 190 and 700 nm. To identify the separated peaks, a number of flavonoids were separated under the same conditions, including 7,4′-dihydroxyflavone, ononin, liquiritigenin, isoliquiritigenin (Indofine Chemicals, Hillsborough, NJ, USA), formononetin, kaempferol (Fluka Chemie, Buchs, Switzerland) and medicarpin (Sequoia Research Products Ltd., Oxford, UK). HPLC of flavonoid extracts was carried out three times each from control and *CHSi* roots, using different batches of transformed plants. All batches yielded similar results.

Phenotypic characterizations

For assays of root growth, lateral development and gall formation, composite plantlets transformed with the pK7GWIWG2D(II) vector were transferred to 15-cm-diameter Fåhraeus plates after they had been screened for GFP fluorescence. For assays of root growth, the position of the root tip was marked on the back of the plate every second day. After 2 wk, the root tip was inoculated with a liquid culture of *S. meliloti*. GFP-labelled *S. meliloti* strain 1021 was inoculated into liquid Bergensen's modified medium and grown overnight to an optical density at 600 nm (OD_{600}) of 0.2 (28°C at 180 rpm), supplemented with 2 μM luteolin (Sigma). The growth of the tip was then monitored for a further 2 wk. After 2 wk, the plates were photographed with a digital camera. Measurements were made from the digital images using the ImageJ software package (http://rsbweb.nih.gov/ij/). Similarly, for assays of lateral root emergence, the root tip and the tips of emerging lateral roots were marked every 2 d over a period of 4 wk before being measured and compiled digitally.

For assays of root gall development, the root tip was inoculated with a suspension of *Me. javanica*. After 4 wk, the numbers of galls on each root were counted. Measurements of root gall width and length were made under a light microscope containing a graticule. Measurements of giant cell area and counts of cortical and pericycle cell types were made from images of cross-sections through the centre of each gall. Digital images were analysed using ImageJ to calculate giant cell areas and count individual cells.

Statistical analysis

Student's *t*-tests and ANOVA were performed using Prism 4 for Macintosh (version 4.0c, GraphPad Software: GraphPad Software, La Jolla, CA, USA).

Results

Flavonoid-deficient roots form lateral roots and galls but not nodules

Our first aim was to determine whether flavonoids are required for the organogenesis of nodules, lateral roots and root galls formed by RKNs. Silencing of *CHS* to produce flavonoid-deficient roots was achieved using a binary RNAi vector from which the target (*CHS*) is expressed as an intron-spliced hairpin RNA (hereafter named *CHSi*). The empty vector control produces a nonsense hairpin. Observations on control hairy roots showed that all three developing organs are highly fluorescent, most probably because of the fluorescence of flavonoids (Fig. 1a,f,k). At least 20 control and 20 *CHSi* roots were sectioned after inoculation with *S. meliloti* to follow nodule development and after inoculation with *Me. javanica* to follow gall development; the lateral root emergence zone of untreated roots was sectioned to observe lateral root formation. We focused on the stages of development involving ongoing cell divisions, at which concurrent flavonoid accumulation

and auxin accumulation have been reported in various species (see Introduction). Cross-sections of control hairy roots during the early stages of development of each of the three organs showed that fluorescent compounds accumulated in the dividing cortical and pericycle cells of young nodule primordia (Fig. 1b,c), in lateral root primordia (Fig. 1g,h), and in dividing pericycle and cortical cells that surround the expanding giant cells of 4-wk-old galls (Fig. 1k,l). The fluorescence was enhanced after staining with the flavonoid-specific reagent diphenylboric acid-2-aminoethyl ester (Peer *et al.*, 2001; Wasson *et al.*, 2006; Buer *et al.*, 2007). Cells in cross-sections of *CHSi* roots did not contain intracellular fluorescent compounds, although cell wall fluorescence was still observed (Fig. 1d,i,n). After inoculation with *S. meliloti*, no cortical or pericycle cell divisions were observed at the spot inoculation site and no nodules were formed in any of the 20 roots analysed (Fig. 1d,e). However, lateral roots and root galls developed on *CHSi* roots. Cross-sections showed that the developing lateral roots (Fig. 1i,j) had a similar morphology to lateral roots forming on control roots, but were not characterized by the accumulation of flavonoids in the developing organ. Galls also formed on *CHSi* roots and giant cells were present in the centre of the galls (Fig. 1n,o), similar to those developing in control roots. Although galls forming on control roots showed several cell layers of dividing pericycle and cortical cells (Fig. 1l,m), divisions in the pericycle layer surrounding galls forming on *CHSi* roots were typically not as obvious (Fig. 1n,o; note that, in some cases, cytoplasmic contents of giant cells were lost on sectioning).

HPLC was used to confirm silencing of the flavonoid pathway in *CHSi* roots in which lateral roots and galls were forming. As shown in Fig. 2, flavonoid extracts of *CHSi* root segments containing emerging lateral roots (Fig. 2a) or root galls (Fig. 2b) were almost devoid of any flavonoid compounds compared with control roots. One absorbing peak was detected in galls of *CHSi* roots (retention time of 19 min in Fig. 2b); however, this peak did not match any of the known flavonoids reported to occur in *M. truncatula* roots (Farag *et al.*, 2007) and currently remains unidentified. Chromatograms of *CHSi* roots inoculated with rhizobia were very similar to those of uninoculated *CHSi* roots forming lateral roots, i.e. no flavonoids were detected (data not shown). We noted that flavonoid-containing samples of uninoculated roots (Fig. 2a) and galls (Fig. 2b) differed in two peaks eluting at 12 and 10 min, respectively. These peaks did not match any flavonoid standards used or any flavonoids identified in a systematic study by Farag *et al.* (2007), but they could be caused by nonflavonoid phenolic compounds that might accumulate differentially in roots and galls (Pi & Rohde, 1967; Pegard *et al.*, 2005).

Having observed that lateral roots and root galls, but not nodules, develop on *CHSi* roots, we characterized the phenotypes of the three organogenesis processes more closely.

Inoculation with *S. meliloti* reduces root growth in flavonoid-deficient, but not control hairy roots

Flavonoid-deficient roots transformed with either the pHellsgate8 vector or the pK7GWIWG2D(II) vector containing a *CHSi* construct showed no cell divisions in either pericycle or cortical cells after inoculation with rhizobia. However, in both types of transformed plant, root hair curling was observed within 48 h (data not shown). Both observations agree with those by Wasson *et al.* (2006) and Zhang *et al.* (2009). Although *CHSi* roots were unable to form nodules, we observed differences in root growth of *S. meliloti*-inoculated control and *CHSi* roots. To quantify this effect, root growth was recorded every 2 d for 14 d. The roots were then inoculated with a liquid culture of *S. meliloti* which had been supplemented with the flavonoid luteolin to induce Nod factor synthesis. There was no significant difference in root length between control and *CHSi* roots at the time of inoculation ($P > 0.05$, $n = 70$–98). However, within 2 d after inoculation, the root growth rate in *CHSi* roots was significantly reduced compared with the rate pre-infection (Fig. 3a, $P < 0.0001$, $n = 70$–98). In comparison, the root growth rate in control hairy roots increased significantly within 2 d after inoculation (Fig. 3b, $P < 0.0001$, $n = 70$–98).

Root growth was recorded for a further 14 d (Fig. 3b). Two-way ANOVA showed that the interaction of flavonoid status and inoculation was statistically significant ($P < 0.05$, $n = 70$–98). In *CHSi* roots, inoculation significantly reduced the average root growth rate from 1.22 to 0.75 mm d^{-1}. In control roots, inoculation did not significantly reduce the root growth rate, with the average growth rate decreasing slightly from 1.34 to 1.24 mm d^{-1}. These results suggest that *CHSi* roots still responded to rhizobia, even though no nodules were formed. The observed reduction in the growth response is opposite to the temporary root growth stimulation in control roots.

Flavonoid deficiency does not influence lateral root formation

The number of lateral roots per hairy root of composite plantlets with *CHSi* and control roots was recorded every 2 d for 4 wk. At the end of that period, there was no statistically significant difference between the numbers of lateral roots forming on *CHSi* and control hairy roots (Fig. 4a, $P > 0.05$, $n = 54$). There was no significant difference in the pattern of emergence of lateral roots over the 4-wk period (Fig. 4b), and no significant difference in the average length of the lateral roots ($P > 0.05$: flavonoid-deficient, 17.56 ± 0.94 mm, $n = 155$; control, 17.61 ± 0.93 mm, $n = 159$).

Effect of flavonoids on gall formation

To assess any differences in root gall formation, *CHSi* and control hairy roots were inoculated with *Me. javanica*. After

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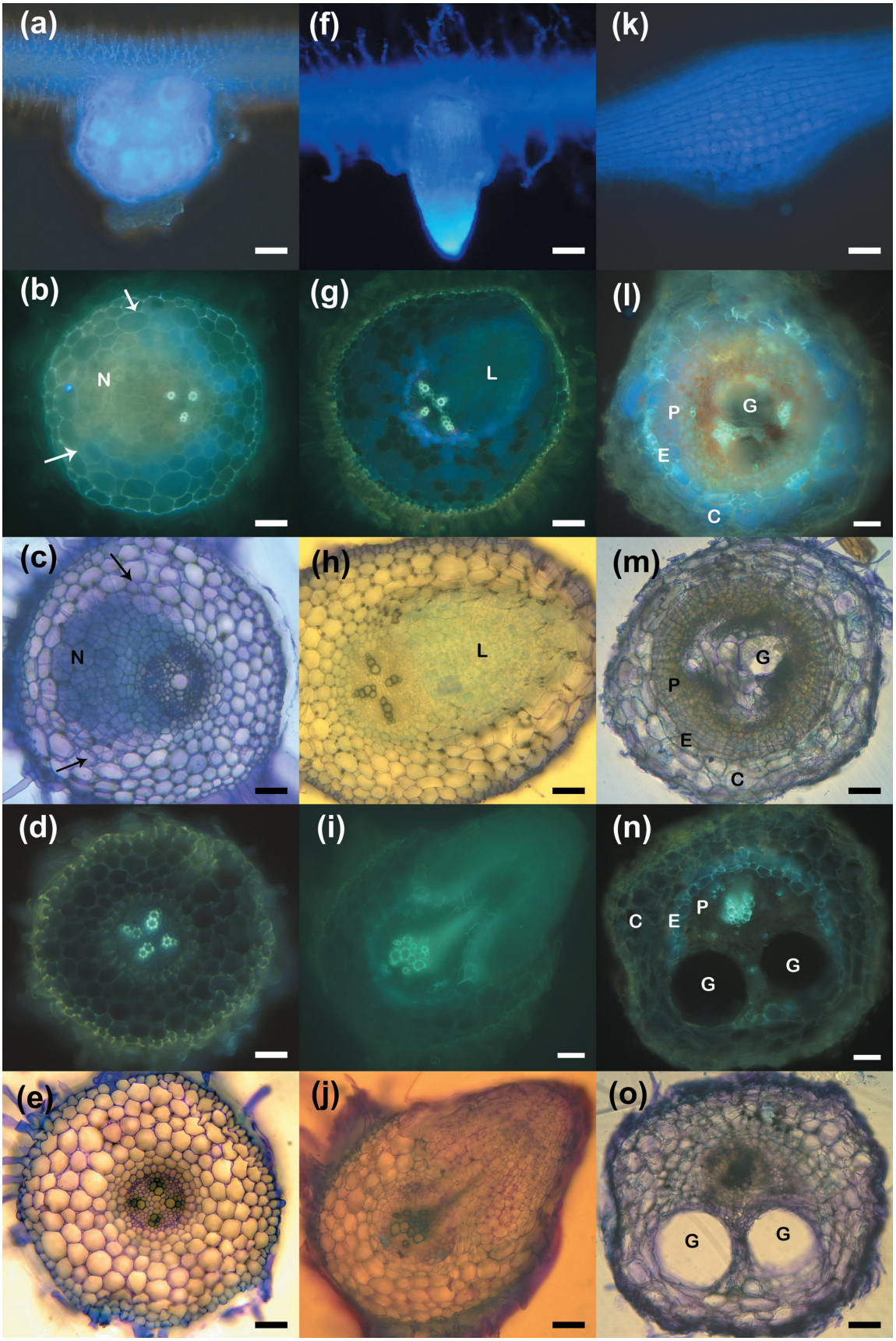

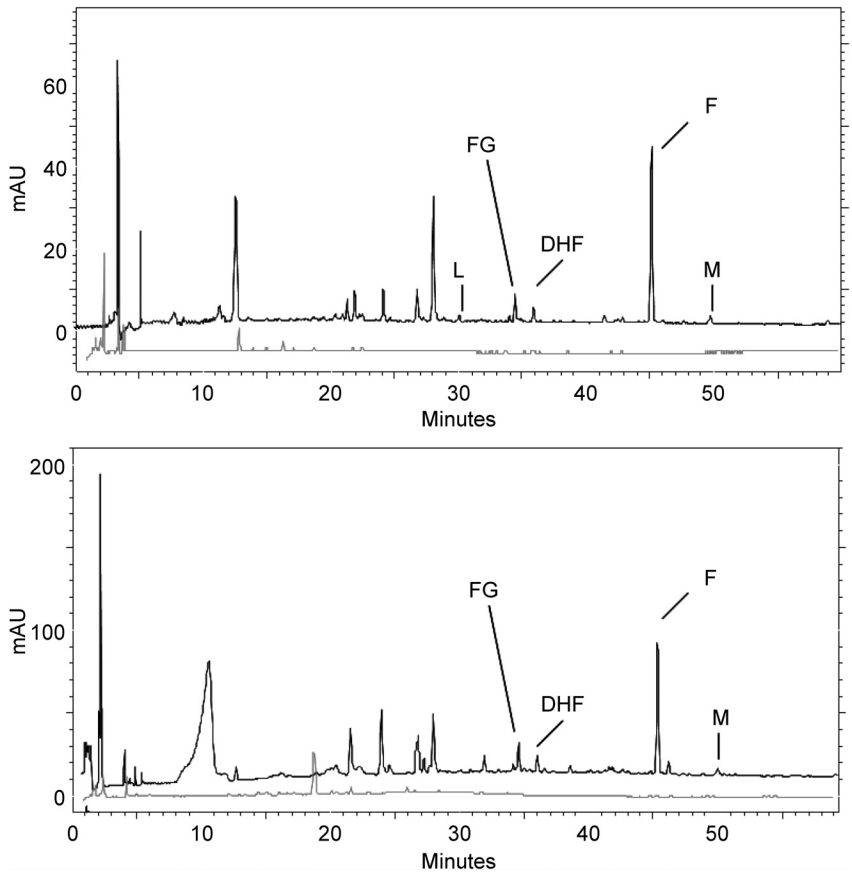

Fig. 2 High-performance liquid chromatograms of flavonoid extracts from roots and galls of *Medicago truncatula*. All chromatograms show absorbance at 300 nm. (a) Chromatograms of root extracts from empty vector control (top black line) and *CHSi* hairy roots (bottom grey line) containing lateral roots of different developmental stages. The two lines were offset by 20 units to distinguish them, but are shown at the same scale. (b) Chromatograms of extracts from 4-wk-old galls growing on empty vector control (top black line) and *CHSi* hairy roots (bottom grey line). DHF, 7,4′-dihydroxyflavone; F, formononetin; FG, formononetin-7-O-β-D-glucoside (ononin); L, liquiritigenin; M, medicarpin.

Fig. 1 Comparison of nodulation, lateral root formation and gall formation in *CHSi* and control hairy roots of *Medicago truncatula*. Cross-sections in the control hairy roots (b, g, l) show intracellular fluorescent compounds that are enhanced by staining with the flavonoid-specific reagent diphenylboric acid-2-aminoethyl ester (DPBA). Fluorescence is still apparent in the *CHSi* roots (d, i, n), but is restricted to the cell walls where nonflavonoid phenolics accumulate and is not enhanced by staining with DPBA. (a) Autofluorescence in a 2-wk-old nodule on a control hairy root. (b) Fluorescence in a cross-section of a nodule primordium (N) developing in a control hairy root 72 h postinoculation (hpi) with *Sinorhizobium meliloti*, after staining with DPBA, which preferentially red shifts flavonoid compounds. Note the orange staining of flavonoids inside the dividing cortical and pericycle cells (between arrows), compared with the blue fluorescence in nondividing cortical cells. (c) Cross-section of a nodule primordium 72 hpi with *S. meliloti* developing in a control hairy root, stained with toluidine blue. (d) Fluorescence in a cross-section of a *CHSi* hairy root 72 hpi with *S. meliloti*, stained with DPBA. Note the absence of intracellular orange or blue fluorescence; fluorescence is restricted to nonflavonoid phenolics in the cell wall. (e) Cross-section of a *CHSi* hairy root 72 hpi with *S. meliloti*, stained with toluidine blue. (f) Autofluorescence in an emerging lateral root on a control hairy root. (g) Fluorescence in a cross-section of an emerging lateral root (L) developing in a control hairy root stained with DPBA. Note the weak orange fluorescence within the cells of the emerging lateral root, compared with the blue fluorescence in the surrounding cortical cells. (h) Cross-section of an emerging lateral root developing in a control hairy root, stained with toluidine blue. (i) Fluorescence in a cross-section of an emerging lateral root developing in a *CHSi* hairy root, stained with DPBA. Note the absence of intracellular blue or orange staining, with fluorescence confined to the nonflavonoid phenolics of the cell walls. (j) Cross-section of an emerging lateral root developing in a *CHSi* hairy root, stained with toluidine blue. (k) Autofluorescence in a 4-wk-old gall on a control hairy root. (l) Fluorescence in a cross-section of a 4-wk-old gall in a control hairy root, stained with DPBA. Note the giant cells (G) and the orange-stained cells in the vascular cylinder, and several layers of divided pericycle cells (P, yellow and green staining) next to the endodermis (E), which shows blue intracellular and strong light blue cell wall fluorescence. Cortical cells (C) outside the endodermis contain blue fluorescence. (m) Cross-section of a 4-wk-old gall in a control hairy root, stained with toluidine blue. (n) Fluorescence in a cross-section of a 4-wk-old gall in a *CHSi* hairy root, stained with DPBA. Giant cells are present but very few pericycle cells have divided. Fluorescence is largely confined to the cell walls, with little intracellular orange or blue fluorescence. (o) Cross-section of a 4-wk-old gall in a *CHSi* hairy root, stained with toluidine blue. Bars: f and k, 300 μm; a, b, d, e, g, i, j, l, m, n and o, 100 μm; c and h, 80 μm.

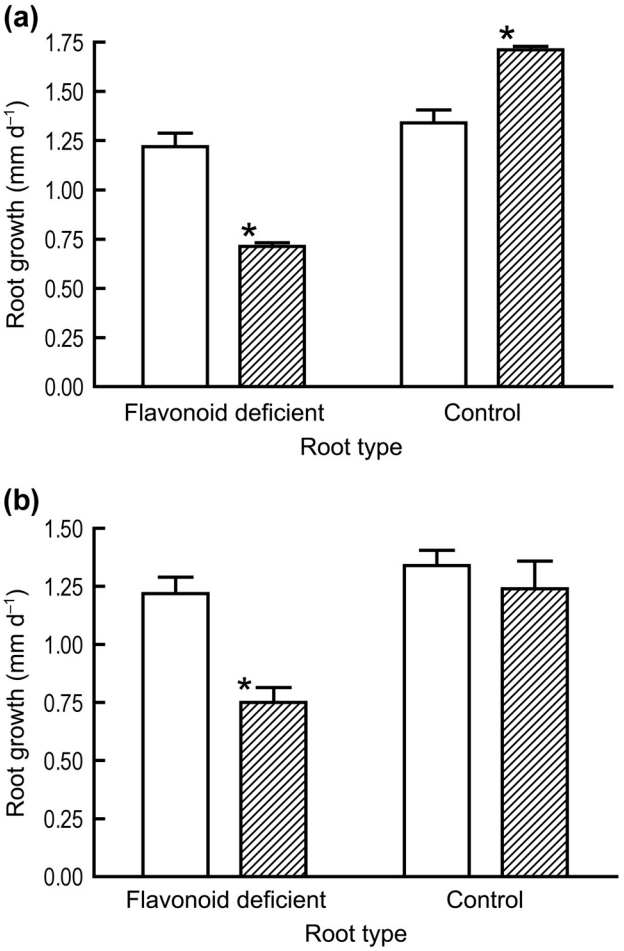

Fig. 3 Root growth measurements in *CHSi* and control hairy roots of *Medicago truncatula* inoculated with *Sinorhizobium meliloti*. (a) Effect of *S. meliloti* on the rate of root growth averaged over the first 48 h after inoculation in *CHSi* and control hairy roots. The asterisk indicates a significant difference between the rate pre- and postinoculation (Student's *t*-test, $P < 0.0001$, $n = 70$–98). (b) Effect of *S. meliloti* on the rate of root growth averaged over 2 wk after inoculation in *CHSi* and control hairy roots (Student's *t*-test, $P < 0.0001$, $n = 70$–98). Error bars indicate SEM.

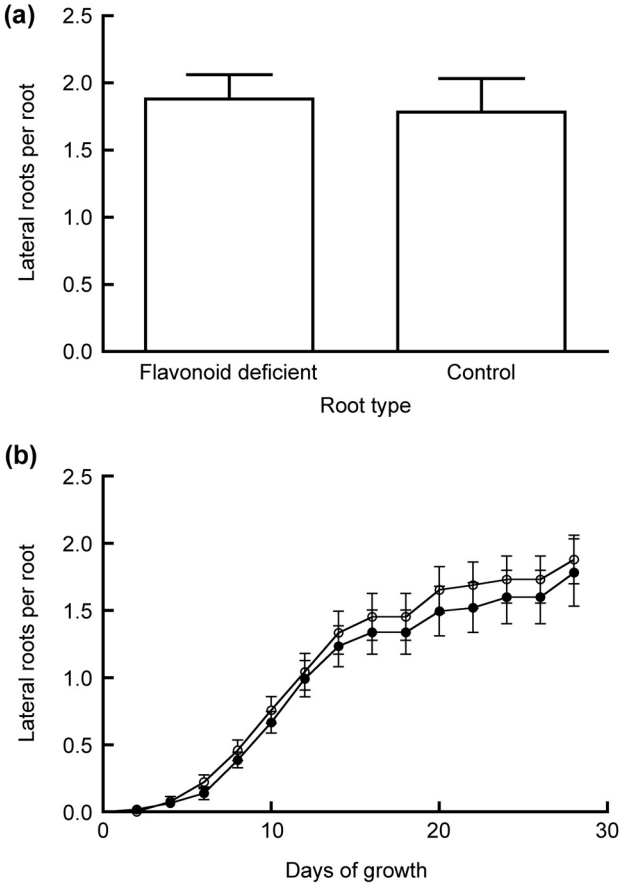

Fig. 4 Lateral root numbers in *CHSi* and control hairy roots of *Medicago truncatula*. (a) The average number of lateral roots forming per hairy root in *CHSi* and control hairy roots (Student's *t*-test, $P > 0.05$, $n = 54$). (b) The average number of lateral roots forming per hairy root, measured every 2 d for 28 d in *CHSi* (open circles) and control (filled circles) hairy roots (Student's *t*-test, $P > 0.05$, $n = 155$–159). Error bars indicate SEM.

4 wk, the numbers of galls on each inoculated root were counted. There was no significant difference in the number of galls forming per hairy root in *CHSi* compared with control roots (Fig. 5a, $P > 0.05$, $n = 53$). Galls forming on *CHSi* roots were not significantly narrower (Fi.g 5b, $P = 0.0901$, $n = 21$) but were significantly shorter (Fig. 5c, $P < 0.05$, $n = 21$) than galls forming on control roots.

Because we had previously observed differences in the number of dividing cell layers in galls formed on *CHSi* and control hairy roots (*cf.* Fig. 1n,o), we quantified the number of divided pericycle and cortex cells, as well as the number and size of giant cells. The number (Fig. 6a) and size (Fig. 6b) of giant cells were not significantly different between *CHSi* and control hairy roots ($P > 0.05$, $n = 22$–36). The strong autofluorescence of the cell wall of the endodermis was used to distinguish between cortical and pericycle cell layers. *CHSi* galls had an average of 51.0 (SEM = 10.9) pericycle and 79.6 (SEM = 8.6) cortical cells per cross-section, whereas control galls had an average of 125.0 (SEM = 8.6) pericycle and 86.2 (SEM = 4.7) cortex cells per cross-section. The ratio of the number of pericycle to cortical cells was significantly lower in *CHSi* than in control galls (Fig. 6c, $P < 0.05$, $n = 5$). Despite the altered morphology, both *CHSi* and control hairy roots formed egg sacks with juveniles that infected subsequently inoculated plants after 6–8 wk. This indicates that viable juveniles can be produced in the absence of flavonoids.

Discussion

The hypothesis tested in this study was that flavonoids are required for the organogenesis of secondary root organs,

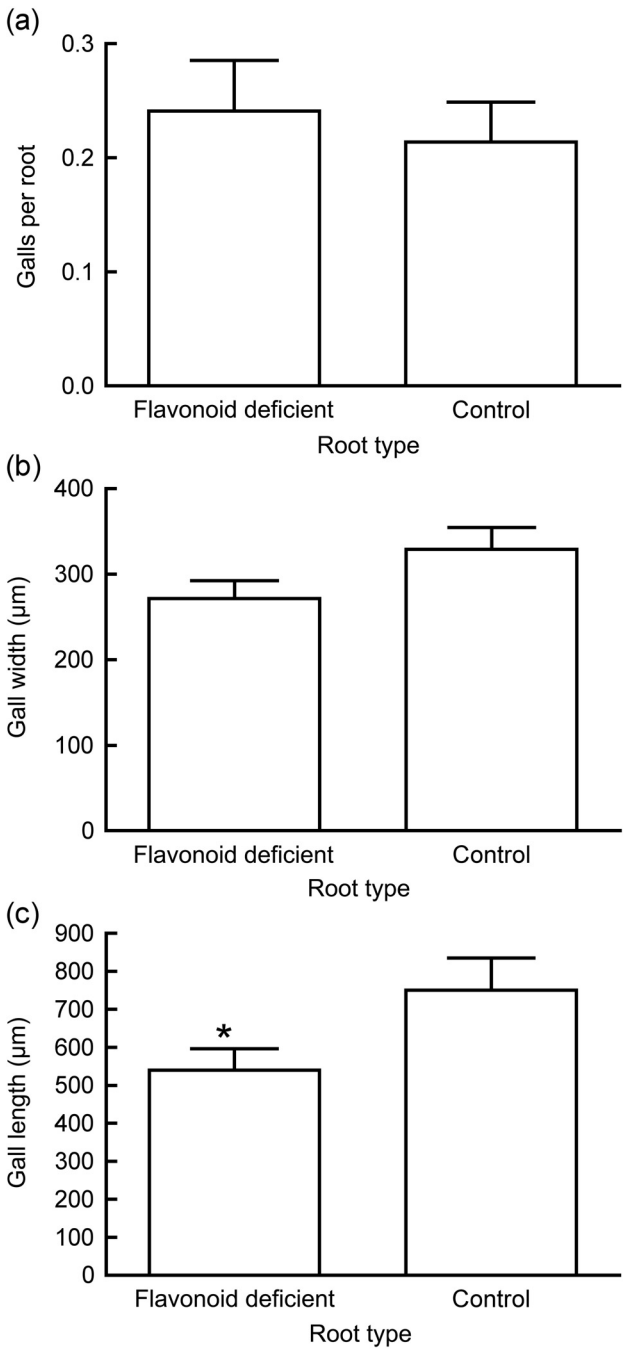

Fig. 5 Effects of chalcone synthase gene (*CHS*) silencing on gall formation in *Medicago truncatula*. (a) The number of galls forming on *CHSi* and control hairy roots (Student's *t*-test, $P > 0.05$, $n = 53$). (b) The average width of galls forming on *CHSi* and control hairy roots. (Student's *t*-test, $P > 0.05$, $n = 21$). (c) The average length of galls forming on *CHSi* and control roots. The asterisk denotes a statistically significant difference between the two root types (Student's *t*-test, $P < 0.05$, $n = 21$). Error bars indicate SEM.

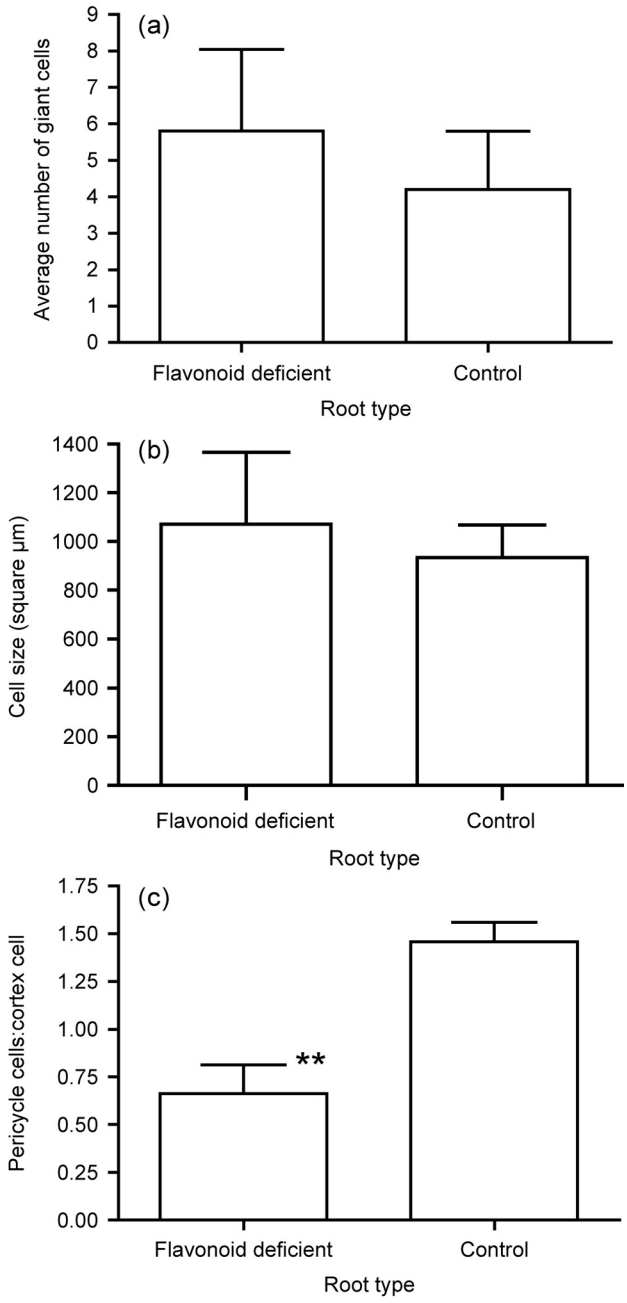

Fig. 6 Effect of chalcone synthase gene (*CHS*) silencing on giant cells and cell divisions in galls. (a) The number of giant cells forming inside galls on *CHSi* and control hairy roots (Student's *t*-test, $P > 0.05$, $n = 5$ roots). (b) The average cross-sectional area of giant cells forming inside galls on *CHSi* and control hairy roots (Student's *t*-test, $P > 0.05$, $n = 22$–36 giant cells). (c) The ratio of pericycle cells to cortex cells in galls developing on *CHSi* and control hairy roots. The asterisk denotes a statistically significant difference between the two root types (Student's *t*-test, $P < 0.05$, $n = 5$ roots). Error bars indicate SEM.

including nodules, lateral roots and root galls. Although flavonoids were required for the initiation of nodules, as reported previously (Wasson *et al.*, 2006; Zhang *et al.*, 2009), they were not required for the organogenesis of lateral roots and root galls. However, our study showed, for the first time, that flavonoids have an effect on the size and cell divisions inside root galls and mediate changes in root growth during nodulation.

The role of flavonoids during nodulation in *M. truncatula*

Flavonoids can have several roles during nodulation. First, flavonoids can act as auxin transport regulators during indeterminate nodule initiation (Wasson *et al.*, 2006; Zhang *et al.*, 2009). Flavonoids also regulate Nod factor synthesis by rhizobia (Redmond *et al.*, 1986; Subramanian *et al.*, 2006, 2007; Zhang *et al.*, 2009). Certain flavonoids have roles in the defence responses of roots to microbes, including rhizobia, and their induction during nodulation may partially serve to regulate the infection of rhizobia, which have been viewed as 'benevolent pathogens' (Djordjevic *et al.*, 1987; Spaink, 1995). In *M. truncatula* inoculated with rhizobia that were pretreated with luteolin to ensure Nod factor synthesis, no nodules were formed, and no pericycle or cortical cell divisions were observed at the inoculation site. However, the roots were still responsive to rhizobia, indicated by root hair curling (this study; Wasson *et al.*, 2006) and changes in root growth. Inoculation of control roots temporarily increased root growth after inoculation. A similar increase in root growth has been observed in *M. truncatula* after inoculation with purified Nod factors (Olah *et al.*, 2005). It is possible that the temporary auxin transport inhibition caused by rhizobia stimulates root growth, similar to the stimulating effect of the synthetic auxin transport inhibitor *N*-1-naphthylphthalamic acid (Fujita & Syono, 1996). By contrast, root growth was inhibited by rhizobia in *CHSi* roots. As rhizobia do not alter auxin transport in *CHSi* roots (Wasson *et al.*, 2006), it is unlikely that this effect on root growth is directly related to auxin transport. Instead, it is possible that the roots divert some energy into the regulation of defence responses against rhizobia using flavonoid-independent defence responses. Alternatively, a lack of flavonoids could have an effect on the auxin content of the roots, because flavonoids also regulate auxin breakdown by IAA oxidases (Furuya *et al.*, 1962; Mathesius, 2001). In Arabidopsis wild-type and flavonoid-deficient *tt4* mutants, similar concentrations of free IAA were measured in inflorescences, although small shifts in IAA accumulation between the apex and base of inflorescences were observed (Brown *et al.*, 2001).

The role of flavonoids during lateral root formation in *M. truncatula*

Lateral root formation is mediated by changes in auxin transport and perception (Fukaki *et al.*, 2007). Auxin transport from the shoot to the root is positively correlated with the emergence of lateral roots (Bhalerao *et al.*, 2002). Therefore, we expected that flavonoid-deficient roots, which show significantly increased auxin transport (Wasson *et al.*, 2006; Zhang *et al.*, 2009), would form more lateral roots than controls. However, this was not the case. Both the morphology and emergence of lateral root formation in *M. truncatula* hairy roots were similar to those of control roots. It is possible that the increase in auxin transport was not sufficient to increase lateral root numbers in *M. truncatula*. Alternatively, altered auxin sensitivity in *A. rhizogenes*-transformed roots may mask subtle lateral root phenotypes (Shen *et al.*, 1988), but control hairy roots were used to control for this possibility. However, although RNAi in *A. rhizogenes* hairy roots of *M. truncatula* has been used to demonstrate lateral root phenotypes (Gonzalez-Rizzo *et al.*, 2006), this is the first time that an auxin-related phenotype has been produced by this method.

The Arabidopsis *tt4* mutant showed increased lateral root density when plants were grown on sucrose-containing medium under continuous light (Brown *et al.*, 2001). By contrast, when the *tt4* mutant was grown with a 16-h or 8-h light period without sucrose on hard slanted agar plates, the lateral root density decreased (Buer & Djordjevic, 2009). It is likely that the carbon supply to the root system and the auxin transport rates interact to determine lateral root density. Our results in *M. truncatula* support the observation in Arabidopsis that flavonoids are not required for the organogenesis of lateral roots. This also suggests that, if lateral root development requires the redistribution of auxin, this is not mediated by flavonoids. It remains elusive why flavonoids accumulate specifically in lateral root primordia in Arabidopsis, *M. truncatula* and other legume species (Mathesius *et al.*, 1998a; Peer *et al.*, 2001; Morris & Djordjevic, 2006). Flavonoids may play a role as antioxidants to protect dividing cells from damage (Rice Evans *et al.*, 1997), but these functions may not directly influence organogenesis.

The role of flavonoids during gall formation in *M. truncatula*

Similar to the accumulation of flavonoids in nodule and lateral root primordia, root galls are characterized by the accumulation of flavonoids in cortical and vascular cells (Hutangura *et al.*, 1999; this study). Flavonoids have been suggested to regulate auxin transport, as their accumulation is observed at the same early stages of gall formation. Similar to lateral root and nodule development, auxin was found to be a crucial regulator of root gall development (Goverse *et al.*, 2000b). However, we found that flavonoid deficiency did not prevent the formation of galls or giant cells. This supports studies in Arabidopsis showing that the flavonoid-deficient *tt4* mutant does not affect RKN reproduction (Wuyts *et al.*, 2006a). Therefore, as in the case of lateral root formation, flavonoids are unlikely to mediate changes in auxin transport or accumulation necessary for gall formation. However, galls of flavonoid-deficient roots were shorter and showed decreased numbers of divided cells in the pericycle, whereas cortical cell numbers were not affected. This could be a direct effect of flavonoids on the division of these cell types, although it is unlikely to be a specific mechanism, as pericycle cell division was not affected by flavonoids during lateral root formation. The lack of flavonoids could also have

indirect effects on gall development because flavonoids act as defence compounds (Kaplan *et al.*, 1980; Chitwood, 2002) and can affect nematode behaviour (Wuyts *et al.*, 2006b).

Giant cells arise as a result of repeated mitosis without cytokinesis, followed by endoreduplication (Jones & Payne, 1978; Goverse *et al.*, 2000a). We found no change in the number or size of giant cells in *CHSi* roots. Endoreduplication in giant cells has been shown to be under the control of the mitotic inhibitor *CCS52A* (Favery *et al.*, 2002), but a requirement for flavonoids as cell cycle regulators has not been demonstrated for giant cells. However, evidence from human and animal studies points to a role for flavonoids as regulators of endoreduplication and the cell cycle (Sato *et al.*, 1994; Cantero *et al.*, 2006). Therefore, it is possible that flavonoids are active cell cycle and endoreduplication regulators in animals, but not in plants.

Outlook

Future studies could be directed at determining which signals are required in the different root organogenesis programmes to regulate auxin transport and accumulation, if not flavonoids. For example, ethylene is another internal regulator of auxin transport and signalling, and is known to interact with auxin in lateral root formation (Ivanchenko *et al.*, 2008; Negi *et al.*, 2008), nodulation (Prayitno *et al.*, 2006) and root gall formation (Glazer *et al.*, 1986). Cytokinin also interferes with auxin homeostasis and has distinct roles during lateral root (Lohar *et al.*, 2004; Laplaze *et al.*, 2007), nodule (Lohar *et al.*, 2004; Gonzalez-Rizzo *et al.*, 2006) and gall formation (Lohar *et al.*, 2004). In addition, the homeodomain gene *KNOX*, a regulator of meristem function expressed during lateral root, gall and nodule development (Koltai *et al.*, 2001), has been shown to affect auxin transport (Tsiantis *et al.*, 1999) as well as cytokinin signalling (Sakamoto *et al.*, 2006), and may link hormone action during all three organogenesis programmes.

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