New Phytologist

Research

Unraveling the effect of arsenic on the model *Medicago–Ensifer* interaction: a transcriptomic meta-analysis

Alejandro Lafuente[1], Patricia Pérez-Palacios[1], Bouchra Doukkali[1], María D. Molina-Sánchez[2], José I. Jiménez-Zurdo[2], Miguel A. Caviedes[1], Ignacio D. Rodríguez-Llorente[1] and Eloísa Pajuelo[1]

[1]Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, C/ Profesor García González 2, 41012 Sevilla, Spain; [2]Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas-CSIC, Grupo de Ecología Genética, c/ Profesor Albareda 1, 18008 Granada, Spain

Author for correspondence:
Eloisa Pajuelo
Tel: +34 954556924
Email: epajuelo@us.es

Received: *29 April 2014*
Accepted: *25 July 2014*

New Phytologist (2015) **205:** 255–272
doi: 10.1111/nph.13009

Key words: arsenic, legumes, metalloid, microarrays, nodulation, pollution, rhizobia, split-root.

Summary

- The genetic regulation underlying the effect of arsenic (As(III)) on the model symbiosis *Medicago–Ensifer* was investigated using a combination of physiological (split-roots), microscopy and genetic (microarrays, qRT-PCR and composite plants) tools.
- Nodulation was very sensitive to As(III) (median inhibitory dose (ID50) = 20 μM). The effect on root elongation and on nodulation was local (nonsystemic). A battery of stress (salt, drought, heat shock, metals, etc.)-related genes were induced. Glutathione played a pivotal role in tolerance/detoxification, together with secondary metabolites ((iso)flavonoids and phenylpropanoids). However, antioxidant enzymes were not activated.
- Concerning the symbiotic interaction, molecular evidence suggesting that rhizobia alleviate As stress is for the first time provided. Chalcone synthase (which is involved in the first step of the legume–rhizobia cross-talk) was strongly enhanced, suggesting that the plants are biased to establish symbiotic interactions under As(III) stress. In contrast, 13 subsequent nodulation genes (involved in nodulation factors (Nod factors) perception, infection, thread initiation and progression, and nodule morphogenesis) were repressed.
- Overexpression of the ethylene responsive factor *ERN* in composite plants reduced root stress and partially restored nodulation, whereas overexpression of the early nodulin *ENOD12* enhanced nodulation both in the presence and, particularly, in the absence of As, without affecting root elongation. Several transcription factors were identified, which could be additional targets for genetic engineering aiming to improve nodulation and/or alleviate root stress induced by this toxic.

Introduction

Arsenic (As) accumulates in the environment as a result of natural and anthropogenic processes (Adriano, 2001; Smedley & Kinniburgh, 2002), causing environmental and health problems (Duker *et al.*, 2005). Effects on plants include reduced seed germination and growth and increased sterility (Smith *et al.*, 2010; Garg & Singla, 2011; Rao *et al.*, 2011). Impaired photosynthesis leds to nutrient deficiencies and chlorosis (Ullrich-Eberius *et al.*, 1989; Mascher *et al.*, 2002; Singh *et al.*, 2006). At the cellular level, As generates reactive oxygen species (ROS) and nitric oxide (NO) (Mascher *et al.*, 2002; Requejo & Tena, 2005; Rao *et al.*, 2011), whereas lipid peroxidation causes membrane damage (Singh *et al.*, 2006; Tuan *et al.*, 2008).

Toxicity depends on the chemical species (As(III)) > (As (V)) > organic species; Finnegan & Chen, 2012). Arsenate, chemically analogous to phosphate, is taken up by plants through root phosphate absorption systems (Shin *et al.*, 2004; Zhao *et al.*, 2009). Once inside the cell, As(V) replaces phosphate in phosphorylation/dephosphorylation reactions (Nemeti *et al.*, 2010; Finnegan & Chen, 2012). Hence, As(V) is rapidly reduced to As(III) by arsenate reductase (Ellis *et al.*, 2006; Duan *et al.*, 2007). Furthermore, As(III) enters the plant via aquaglyceroporins (Meharg & Jardine, 2003; Bienert *et al.*, 2008; Isayenkov & Maathuis, 2008; Ma *et al.*, 2008; Zhao *et al.*, 2009). Arsenite has a great affinity for thiol groups of proteins and inhibits enzyme activities (Finnegan & Chen, 2012). This high affinity is the key for As (III) detoxification in plants: after complexation with phytochelatins (PCs) and glutathione (GSH), it is transported into the vacuoles (Pickering *et al.*, 2000; Schmöger *et al.*, 2000; Liu *et al.*, 2010) by the multidrug resistance-associated proteins MRP/ABCC1 and MRP2/ABCC2 transporters at the tonoplast (Song *et al.*, 2010; Mendoza-Cózatl *et al.*, 2011). A small fraction of the As is uploaded to the xylem, mediated by the silicon efflux transporter Lsi2 (Ma *et al.*, 2008), and translocated to the shoot. Other detoxification mechanisms include efflux of As(III) from the roots (Xu *et al.*, 2008), a process that is unclear (Smith *et al.*, 2010). Moreover, methylated As species are found in plants, but their presence

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

seems to be associated with microbial reactions (Raab *et al.*, 2005; Lomax *et al.*, 2012). Arsenic also induces complex gene regulation mediated by micro-RNAs (Srivastava *et al.*, 2013).

The legume–rhizobia interaction has attracted attention as a consequence of its use in metal(loid) phytostabilization in polluted soils (Pajuelo *et al.*, 2011). Legumes combine moderate tolerance, accumulation in roots, low translocation to green tissues and the ability to grow without additional nitrogen supply (Pajuelo *et al.*, 2007; Reichman, 2007; Dary *et al.*, 2010). However, it is important to determine the effect of metalloids on the symbiosis. Metal(loid)s decrease the biodiversity and activity of microbial populations in soils, selecting resistant populations (Lakzian *et al.*, 2002; Broos *et al.*, 2005; Wang *et al.*, 2011). Arsenic resistance mechanisms in bacteria include complexation with glutathione/metallochaperones, efflux from the bacterial cell, arsenite oxidation, anaerobic arsenate reduction, and methylation (Bhattacharjee & Rosen, 2007).

Regarding legumes, many studies have reported that even low As concentrations lead to a decrease in the number of nodules, the efficiency of nodulation and/or symbiotic nitrogen fixation (Reichman, 2007; Pajuelo *et al.*, 2008; Vázquez *et al.*, 2008; Talano *et al.*, 2012), which has been attributed to reduced rhizobial infections (Pajuelo *et al.*, 2008) or bacterial mobility (Talano *et al.*, 2012). However, data on the genetic basis of this behaviour are scarce. Lafuente *et al.* (2010) reported low expression levels of several nodulin genes and reduced nodulation in the presence of As. Nevertheless, a global transcriptomic analysis has never been performed in legumes, although many transcriptomic studies have been carried out in nonlegumes, such as *Arabidopsis thaliana* (Abercrombie *et al.*, 2008), rice (*Oryza sativa*; Chakrabarty *et al.*, 2009; Huang *et al.*, 2012; Tripathi *et al.*, 2012a) and *Brassica juncea* (Srivastava *et al.*, 2009). In this work, several physiological and genetic investigations were performed in order to examine the effect of arsenite on nodulation, using the model legume *Medicago truncatula* and *Ensifer* (syn. *Sinorhizobium*) *medicae* MA11. *Ensifer medicae* is considered a better symbiont for *M. truncatula*, compared with *Ensifer meliloti* 1021 (Terpolilli *et al.*, 2008). Moreover, *E. medicae* is more resistant to As (up to 10 mM As(III)) than *E. meliloti* (up to 1 mM) (Yang *et al.*, 2005; Pajuelo *et al.*, 2008); thus, the bacterial sensitivity to As will not be the limiting factor in the legume–rhizobia interaction.

Materials and Methods

Plant growth conditions

Medicago truncatula (Gaertn.) (cv Jemalong) seeds were sterilized and pregerminated as described previously (Lafuente *et al.*, 2010). Seedlings (0.5–1 cm root) were transferred to 1.5% Buffered Nodulation Medium (BNM)-agar medium (Ehrhardt *et al.*, 1992) with or without 25 μM sodium arsenite and inoculated with 100 μl of an overnight culture of *E. medicae* MA11 (*c.* 10^8 colony forming units ml^{-1}). Plates were incubated at 22°C : 16°C and with light : dark 16 h : 8 h, with roots protected from light.

Median inhibitory dose (ID50)

The effect of As(III) on germination, growth, nodulation and chlorophyll content was evaluated using the median inhibitory dose (ID50), which is the As(III) concentration needed to reduce a particular parameter by half. For germination, sterilized seeds were placed in 1.5% water-agar plates with increasing sodium arsenite (0–200 μM). Germination was evaluated after 48 h. For growth parameters and chlorophyll, noninoculated plants were grown in plates containing BNM medium supplemented with 2 mM nitrate and arsenite (0–200 μM). After 20 d, shoot and root length and biomass, and chlorophyll content (Harborne, 1984) were determined. For nodulation, pregerminated seeds were transferred to BNM-agar medium (without nitrogen) with 0–35 μM sodium arsenite and inoculated with MA11. Nodules were counted at 28 d post-inoculation (dpi).

Split-root system

A split-root system was used to determine whether the effect of As on nodulation was local or systemic (see Supporting Information Fig. S1 for a complete description). One of the root units (the noninoculated half) was grown in BNM with 25 μM As(III). The other half was grown in BNM without As and inoculated with MA11. No nitrogen was added to either of the halves, in order to avoid its inhibitory effect on nodulation. At 28 dpi, root elongation and nodulation were evaluated.

Microscopy analysis of infection

Medicago truncatula plants were grown in the presence or absence of 25 μM As(III) and inoculated with *lacZ*-labelled MA11. At 5 dpi, roots were harvested, fixed and stained with X-gal for microscopy observation (Pajuelo *et al.*, 2008).

RNA extraction and microarray hybridization

Plants were grown in four different conditions: noninoculated roots in the absence of As; inoculated roots in the absence of As; noninoculated roots in the presence of 25 μM As(III); and inoculated roots in the presence of 25 μM As(III). Root RNA was extracted from 5-dpi plants using TRIzol reagent (Sigma) and 20 μg was used for cDNA synthesis and Cy3- or Cy5-labelling, using the BioScript Reverse Transcriptase Kit (Bioline, London, UK), according to the manufacturer's instructions. After RNA hydrolysis with 0.2 M NaOH and purification using CyScribe GFX columns (GE Healthcare, Little Chalfont, UK), the labelling efficiency was checked (Küster *et al.*, 2004). Before hybridization, microarray slides were washed for 5 min in 0.1% (v/v) Triton X-100, twice for 2 min in 0.01% (v/v) HCl, for 10 min in 0.1 M KCl and for 1 min in MiliQ water. Slides were blocked for 15 min in QMT Blocking Solution (Quantifoil, Groβlöbichau, Germany) containing 0.02% (v/v) HCl, rinsed in MiliQ water for 1 min and dried by centrifugation (380 ***g*** for 3 min at room temperature). Hybridization was performed in a hybridization cassette (Arrayit Corp., Sunnyvale, CA, USA) in a sample volume

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

of 44 µl DIG Easy Hyb solution (Roche) containing 1 µl of salmon sperm DNA (Invitrogen). Samples were denatured for 5 min at 65°C before injection. After 18 h of hybridization at 42°C, slides were washed twice (for 1 and 5 min, respectively) in 2× SSC and 0.2% (w/v) SDS at 42°C; twice in 0.2× SSC and 0.1% (w/v) SDS at room temperature, both for 1 min; twice (for 2 and 1 min, respectively) in 0.2× SSC solution at room temperature, and once in 0.05× SSC solution at 4°C for 1 min. Finally, slides were dried by centrifugation as described above in this section.

Microarray analysis

Mt16kOLI1Plus microarrays (Hohnjec *et al.*, 2005) contain 16 086 70-mer oligonucleotide probes representing all tentative consensus sequences (TCs) of the TIGR *M. truncatula* Gene Index 5 plus 384 transcription factors and regulators (Moreau *et al.*, 2011) (available at EMBL-EBI: www.ebi.ac.uk/arrayexpress/; accession number A-MEXP-138). Slides were scanned using the Axon GenePix 4100A scanner and the GenePix Pro 6.0 software (Molecular Devices, Silicon Valley, CA, USA), following the manufacturer's directions (Verdnik, 2004). Data files were analysed using EMMA 2.0 software (Dondrup *et al.*, 2003), and spots analogous to background intensity levels or artifacts were discarded. Locally weighted scattered-plot smoothing (LOWESS) normalization was performed using a floor value of 20 and regulated genes were identified using *t*-statistics. To guarantee statistically significant differences, strong restrictions were imposed: (1) at least eight out of 12 signals corresponding to the same gene (3 replicates × 2 biological samples × 2 spots in each array) had to fulfill the following criteria; (2) the deviation of the signals had to be below $P \leq 0.05$; and (3) all genes whose M values were between −1 and +1 were discarded, M being the binary logarithm of the intensity ratio (I) in both channels ($M = \log_2(\mathrm{Ch1}\,I/\mathrm{Ch2}\,I)$). Genes were regarded as significantly overexpressed ($M \geq 1$; $P \leq 0.05$) or significantly inhibited ($M \leq -1$; $P \leq 0.05$) when their expression was ≥ 2-fold or ≤ 2-fold the expression level in control conditions, respectively. Functional visualization of gene expression was performed with MapMan (Thimm *et al.*, 2004; Usadel *et al.*, 2005), adapted for *Medicago* genes by Goffard & Weiller (2006).

Real time qPCR

qRT-PCR was performed for 13 genes using the primers shown in Table S1. Three independent RNA extractions of two independent biological samples were prepared from 5-dpi inoculated roots grown in the presence or the absence of 25 µM As(III), using the RNeasy Plant Minikit (Qiagen, Venlo, the Netherlands). Genomic DNA removal and reverse transcription were performed using the QuantiTect Reverse Transcription Kit (Qiagen). qPCR was performed as described previously (Lafuente *et al.*, 2010), using three constitutively expressed genes (*Msc27* (Microcallus suspension gene-27), *γ-tubulin* and the translation initiation factor *5A-2*) for normalization (Gallardo *et al.*, 2007; Lafuente *et al.*, 2010).

Overexpression of selected genes in composite plants

Generation of *Medicago truncatula* composite plants expressing the ethylene responsive factor *ERN* and the early nodulin *ENOD12* in roots The *ERN* and *ENOD12* genes from *M. truncatula*, amplified with primers described in Table S1, were cloned into pMF-2 (Merchan *et al.*, 2007) and electroporated into *Agrobacterium rhizogenes ARquaA*1 (Boisson-Dernier *et al.*, 2001). The empty vector was transformed as a control. After selection for kanamycine resistance (25 µg ml^{-1}), the positive clones were confirmed by PCR.

Medicago truncatula seedlings (n = 30) were cut *c.* 5 mm from the root tip and inoculated with *A. rhizogenes* carrying pMF2, pMF2-ERN or pMF2-ENOD12. Inoculated seedlings were placed in square plates containing Fahraeus medium (Fahraeus, 1957) with kanamycine (25 µg ml^{-1}) for 3 d, and subsequently transferred to plates with BNM medium, containing or not containing 25 µM sodium arsenite and inoculated with 100 µl of MA11. After incubation at 22°C : 16°C with light : dark 16 h : 8 h for 1 month, root length inhibition and nodule number were determined in individual plants. Results were computed in plants with positive PCR amplification (n = 19–25).

Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2007 and PASW 18 (IBM SPSS Statistic, Armonk, NY, USA). For physiological results and nodulation, data are mean ± SE for 50 plants. For qRT-PCR, data are mean ± SE for three replications of two independent biological samples. Significant differences at $P < 0.05$ are indicated by different letters in Table 2 and Fig. 10.

Results

Response of the *Medicago truncatula–Ensifer medicae* interaction to arsenite

The effect of arsenite was determined using the ID50 (Table 1). Germination was less affected by As(III) compared with growth parameters and nodulation. In general, shoot growth was severely affected, although chlorophyll content remained high at the concentrations used. In particular, the number of nodules was very

Table 1 Median inhibitory concentration (ID50) for nodulation, growth and physiological parameters of *Medicago truncatula* with the microsymbiont *Ensifer medicae* MA11 in the presence of arsenic (As(III))

Parameter	ID50 for As(III) (µM)
Seed germination (48 h)	125
Shoot length (20 d)	40
Shoot biomass (20 d)	60
Chlorophyll (20 d)	> 125
Root length (20 d)	50
Root biomass (20 d)	80
Number of nodules (28 d)	20

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

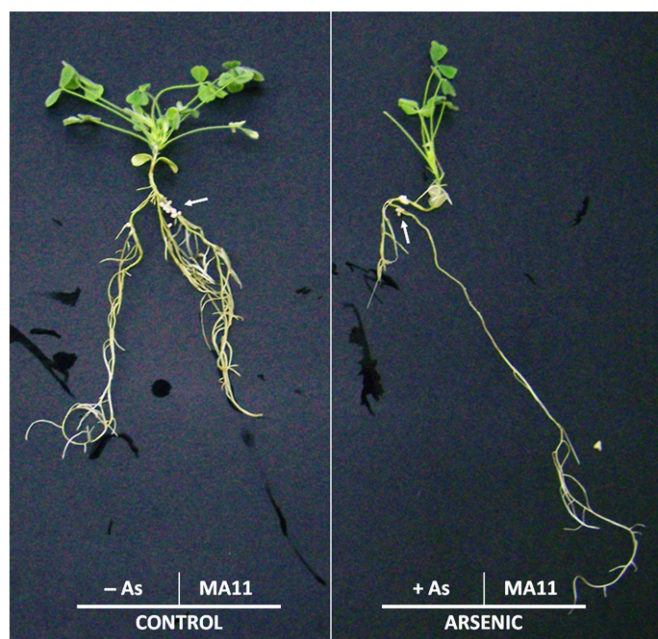


Fig. 1 Effect of arsenic (As) on nodulation and root growth of *Medicago truncatula* plants inoculated with *Ensifer medicae* MA11 determined in a split-root system. CONTROL, half of the root system was grown in the absence of 25 μM As(III) (–As), and the other half was inoculated with *E. medicae* MA11 and grown in the absence of arsenite (MA11). ARSENIC, half of the root system was grown in the presence of As (+As), and the other half was inoculated with *E. medicae* MA11 and grown in the absence of arsenic (MA11). Arrows indicate the nodules.

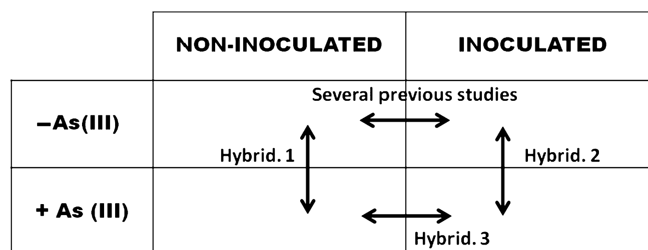


Fig. 2 Scheme depicting the three hybridization developed during this work: hybridization 1 compares non-inoculated roots grown in the presence vs the absence of As(III). Hybridization 2 compares inoculated roots grown in the presence vs the absence of As. Hybridization 3 compares inoculated vs non-inoculated roots, both in the presence of As.

Table 2 Results for the split-root system developed to analyse the effect of arsenic on nodulation and root growth of *Medicago truncatula* plants inoculated with *Ensifer medicae* MA11

	Control		Arsenic	
	−As	MA11	+As	MA11
Root length	9.1 ± 0.7^a	9.4 ± 0.8^a	5.2 ± 0.4^a	12.7 ± 1.1^b
Number of nodules	0	2.8 ± 0.2^a	0	3.0 ± 0.4^a

In the control, half of the root system was grown in the absence of arsenic (without inoculation) and the other half was inoculated (in the absence of As). In the arsenic treatment, half of the root system was grown in the presence of 25 μM As(III) (without inoculation) and the other half was inoculated (in the absence of As). Twenty-eight days after inoculation, plants were harvested and the root length and the number of nodules were determined. Data are mean ± SE ($n = 20$ plants). Different letters indicate significant differences at $P < 0.05$.

sensitive to low As doses (ID50 20 μM), indicating that nodulation was the most sensitive process.

A hydroponic split-root system was developed, in which half of the root was incubated in the presence of arsenite, and the other half was inoculated. Results (Fig. 1, Table 2) showed strong root growth inhibition in the exposed half, whereas the unexposed half grew normally, indicating a local effect of As(III) on root elongation. Moreover, the presence of As(III) in one side did not affect the number of nodules induced in the other side, suggesting a local (nonsystemic) effect of arsenite on nodulation.

Transcriptomic analysis of the interaction of *Medicago truncatula–Ensifer medicae* in the presence of arsenic

In order to identify the genes modulated by As(III) during the rhizobia–legume interaction, three hybridizations were performed (depicted in Fig. 2). Hybridization 1 compared noninoculated plants grown in the presence versus the absence of As. Hybridization 2 compared inoculated plants grown in the presence versus the absence of As. Hybridization 3 compared inoculated and noninoculated plants, both grown in the presence of As. A fourth hybridization (inoculated versus noninoculated plants in the absence of As) was performed previously (i.e. Mitra *et al.*, 2004; Lohar *et al.*, 2006).

Hybridization 1 This hybridization identified genes involved in the response to As(III), independently of nodulation. On the basis of the very restrictive conditions for microarray analysis, 5097 genes were discarded. A total of 263 genes showed increased expression levels (≥ 2-fold) and 528 genes displayed reduced expression levels (≤ ½-fold) in the presence of As(III). The complete list of affected genes is available in the EMBL database (access number E-MTAB-1723), whereas Table S2 shows genes up- or down-regulated more than 5-fold. Genes were manually classified in seven function categories (Fig. 3a). Among the over-expressed genes, the most remarkable finding was the high abundance (20%) of stress response genes, particularly those involved in the response to abiotic stress (82% of them). These genes included those encoding peroxidase ATP5a, glutathione-S-transferase (GST), a germin-like protein similar to that induced by As in rice (Tripathi *et al.*, 2012a) and 1-pyrroline-5-carboxilate synthase (P5CS) involved in proline synthesis, an amino acid involved in stress responses (Szabados & Savouré, 2010). Among the repressed genes, those involved in cell wall architecture constituted 11%, including genes encoding pectin-esterase inhibitors, extensins and cell-wall specific peroxidases. Substantial remodelling of the cell wall is known to occur upon metal stress, with the purpose of strengthening the cell wall and protecting cells against metals (Passardi *et al.*, 2005; Krzesłowska, 2011). Other inhibited genes were those related to photosynthesis, including the gene encoding the RuBisCo small subunit, although the significance of these results in roots is unknown.

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

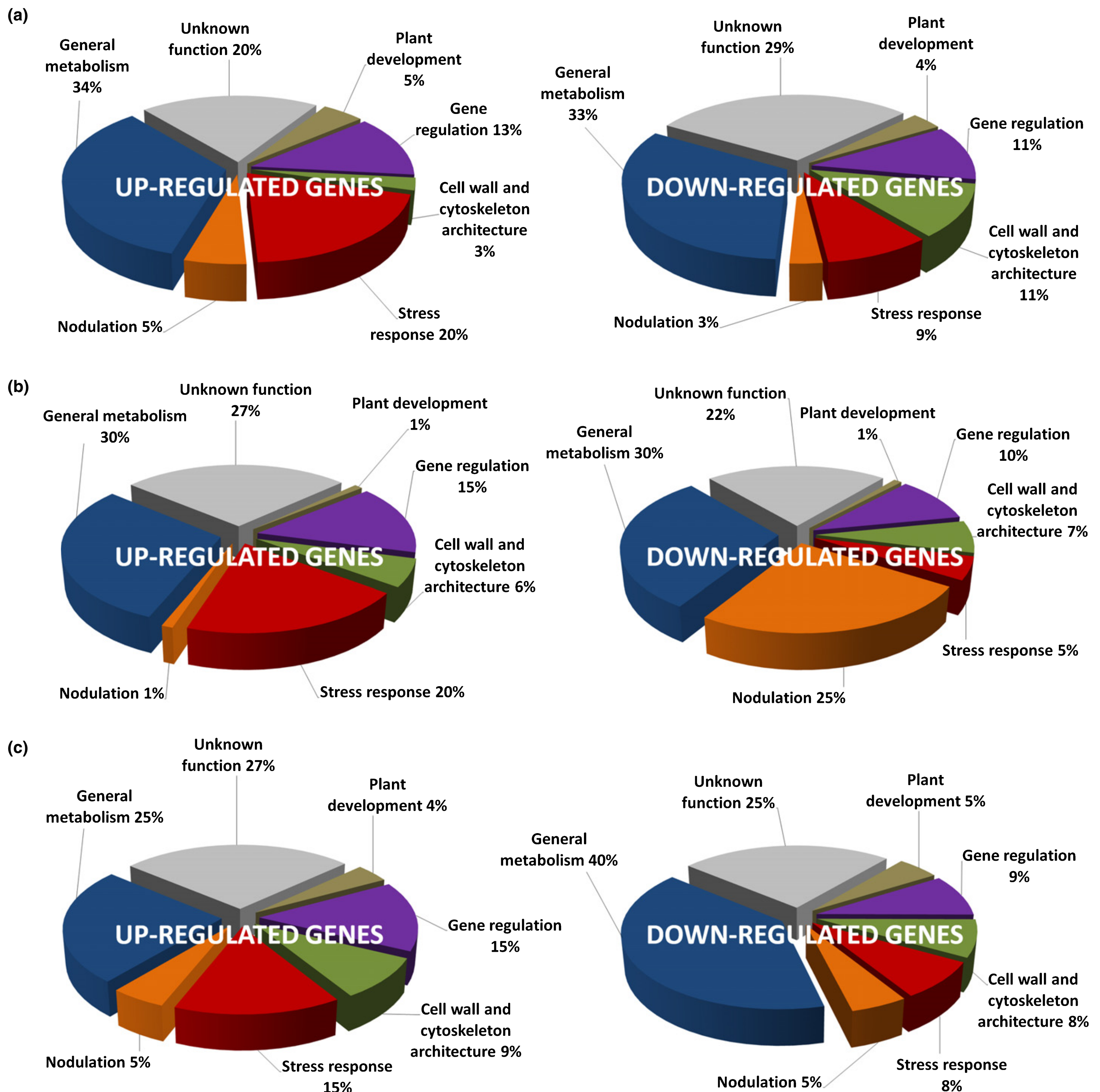


Fig. 3 Manual classification of transcription profiling data from *Medicago truncatula* for hybridizations 1 (a), 2 (b) and 3 (c). Seven different categories were established for both up- and down-regulated genes: general metabolism, gene regulation, cell wall and cytoskeleton architecture, plant development, nodulation, stress response, and unknown function.

It is worth noting that, in spite of plants being not inoculated, 5% of overexpressed genes were related to nodulation, in particular, isoflavone 7-O-methyl transferase and chalcone synthase (8.4-fold and 6-fold induced, respectively). These genes are involved in the synthesis of (iso)flavonoids, molecules that act as signals for plant–bacteria recognition, and may reflect the tendency of the plant to establish symbiotic relationships under stress conditions. By contrast, nodulation genes involved in later events were repressed (a nodule-specific cysteine-rich peptide 62 involved in nodule morphogenesis, and the early nodulins from *Medicago truncatula MtN12A* (nodulin 12A), *MtN12B* (nodulin 12B) and *MtN22* (nodulin 22). These results suggested that, even though the plant secreted flavonoids for plant–bacteria recognition, nodulation was not further prioritized.

A deeper analysis was performed in order to identify metabolic pathways affected by As(III). In addition to being one

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

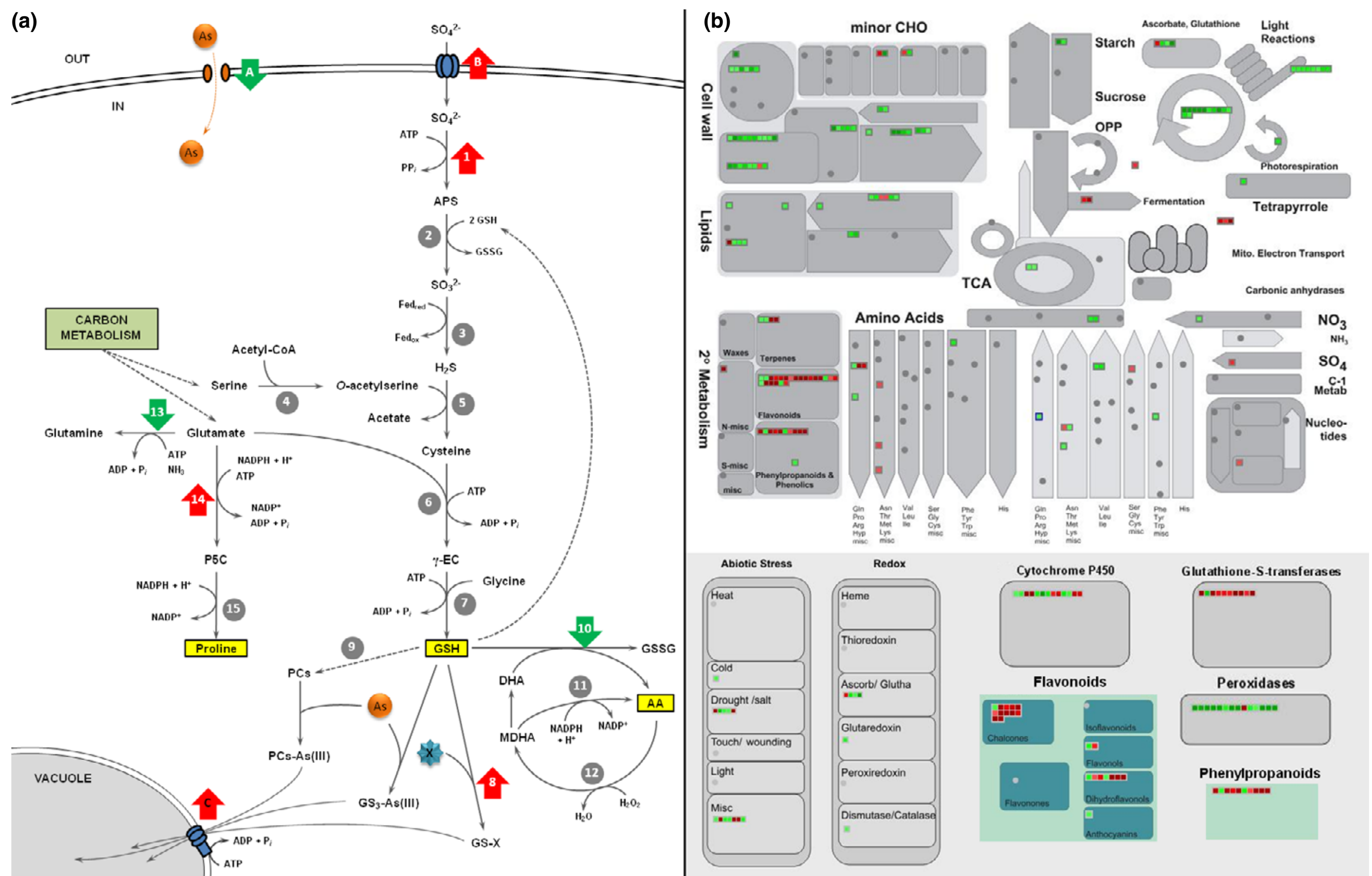


Fig. 4 Most relevant results in hybridization 1 (noninoculated *Medicago truncatula* in the presence versus the absence of arsenic (As)). (a) Pathway showing sulphate assimilation, glutathione and proline synthesis, xenobiotic complexation and the glutathione-ascorbate cycle (modified from Rausch & Wachter, 2005 with permission). The numbers and letters indicate the enzymes and transporters involved in this pathway: A, aquaporin; B, sulphate high-affinity transporter; C, vacuolar ABC transporter (ATP-binding cassette transporter); 1, ATP sulphurylase; 2, APS (adenosin 5′-phosphosulfate) reductase; 3, sulphite reductase; 4, serine transacetylase; 5, O-acetylserine(thiol)lyase; 6, γ-glutamylcysteine synthetase; 7, glutathione synthetase; 8, glutathione transferase; 9, phytochelatin synthase; 10, dehydroascorbate reductase; 11, monodehydroascorbate reductase; 12, ascorbate peroxidase; 13, glutamine synthetase; 14, pyroline-5-carboxilate synthetase; 15, pyroline-5-carboxilate reductase; AA, ascorbic acid; As(III), arsenite; X, xenobiotic. Red up-arrows indicate over-expressed genes. Green down-arrows indicate repressed genes. (b) Diagram showing the MapMan analysis of the transcript data. Above, general metabolism overview. Below, abiotic stress and redox response, phenylpropanoid and flavonoid metabolism, and glutathione S-transferase, cytochrome P450 and peroxidase enzyme families. AA, ascorbic acid; DHA, dehydroascorbate; gamma-EC, gamma glutamyl cysteine; GSH, reduced glutathione; GSSG, oxidized glutathione; MDHA, monodehydroascorbate; PC, phytochelatin; P5C, pyroline-5-carboxylate.

of the main ROS-scavenging metabolites in plants, glutathione is the precursor of phytochelatins, the main peptides involved in complexation of As(III) (Briat, 2010). Synthesis of glutathione is strongly dependent on sulphur (S) supply; hence, the pathway of sulphate assimilation was activated (Fig. 4a), including a high-affinity sulphate transporter and ATP-sulphurylase, which are known to be involved in As tolerance and accumulation (Wangeline *et al.*, 2004; Nocito *et al.*, 2006; Reid *et al.*, 2013). Moreover, the ABC-type transporter involved in the transport of glutathione complexes into the vacuole (Ghosh *et al.*, 1999) was activated. This is the main detoxification pathway for As(III) (Zhao *et al.*, 2009). By contrast, the silicon aquaporin at the plasma membrane, NIP2-1 (Numerous Infections and Polyphenolics 2-1), which is similar to Lsi1 (Low Silicon transporter 1) in rice (Ma *et al.*, 2006), was 2.1-fold down-regulated. This aquaporin represents the main entrance mechanism of arsenite into the cell (Ma *et al.*, 2008), indicating that the plant blocked arsenite entry into roots.

The bioinformatic tool MapMan was used to find pathways co-regulated by As. Some genes involved in cell wall architecture were repressed, as well as those involved in the light and dark reactions of photosynthesis, and starch metabolism (Fig. 4b). We investigated whether the network of redox enzymes involved in ROS scavenging may be affected. Genes involved in the ascorbate–glutathione cycle were down-regulated, including glutathione peroxidase, dehydroascorbate reductase, ascorbate oxidase, ascorbate oxidase promoter-binding protein (AOBP) and chloroplastic superoxide dismutase (between 2- and 3-fold repressed). Other enzymes involved in this cycle, such as glutathione reductase and monodehydroascorbate reductase, did not show significant changes. In addition, several peroxidases involved in H_2O_2 scavenging were 4–5-fold down-regulated. These results suggested that antioxidant enzymes are not involved in the arsenite response in

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

this plant, similar to findings reported in rice exposed to As(V) and As(III) (Chakrabarty *et al.*, 2009), where only the oxidized species As(V) induced genes involved in oxidative stress. By contrast, previous studies in maize (*Zea mays*; Requejo & Tena, 2005) showed similar stress regulation by As(V) and As(III).

In contrast to antioxidant enzymes, genes involved in secondary metabolism were highly induced by As(III) (Fig. 4b), in particular several genes involved in the synthesis of (iso)flavonoids (i.e. isoflavone-7-O-methyltransferase, glucosyltransferase, O-diphenol-O-methyltransferase, naringenin chalcone synthase and the SRG1 (SENESCENCE-RELATED GENE 1) protein). In addition to their role in the plant–bacteria interaction, flavonoids contain aromatic rings involved in ROS scavenging (Pietta, 2000; Michalak, 2006). Phenylpropanoid synthesis was also enhanced (for example, N-hydroxycinnamoyl/benzoyl transferase and O-diphenol-O-methyl transferase were up-regulated); this pathway was induced in plants by abiotic and biotic stresses, such as ozone exposure, low nutrient concentration, and pathogen or herbivore attack (Vogt, 2010).

Hybridization 2 Hybridization 2 identified genes involved in the effect of As(III) on the symbiotic interaction. On the basis of the restrictive criteria, 4177 genes were discarded, 124 genes were over-expressed and 128 genes were inhibited (access number E-MTAB-1723). Table S3 shows genes over-expressed or down-regulated by more than 4-fold. Manual classification (Fig. 3b) showed significant differences in genes involved in stress response and nodulation. After comparison of hybridizations 1 and 2 (Fig. 3a,b), much fewer stress genes were found to be induced (29 versus 62). These results indicated protection, conferred by the inoculum, from the stress caused by As(III) in roots. Induced genes (Table S3) included a sulphate transporter (2.2-fold), GST (5.9-fold) and an ABC transporter (2.4-fold), all of which are involved in As detoxification. Furthermore, the gene coding for Rab genarylgeranyl transferase, which is involved in the cadmium (Cd) response, and Nramp1 (natural resistance-associated macrophage protein 1), which is a root-specific metal transporter (Xiao *et al.*, 2008), were up-regulated by 2–2.5-fold. Other stress-related genes were those encoding P5CS (which is involved in proline synthesis), type A cytochrome P450 monooxigenase, NADP-dependent oxidoreductase P2 (which has activity against toxic substrates and participates in plant antioxidant defence) and STO (which is involved in salt tolerance), and other genes related to cold acclimation or heat shock. In contrast to those involved in abiotic stress, whose expression was generally enhanced, genes related to biotic stress were in general inhibited (Table S3),

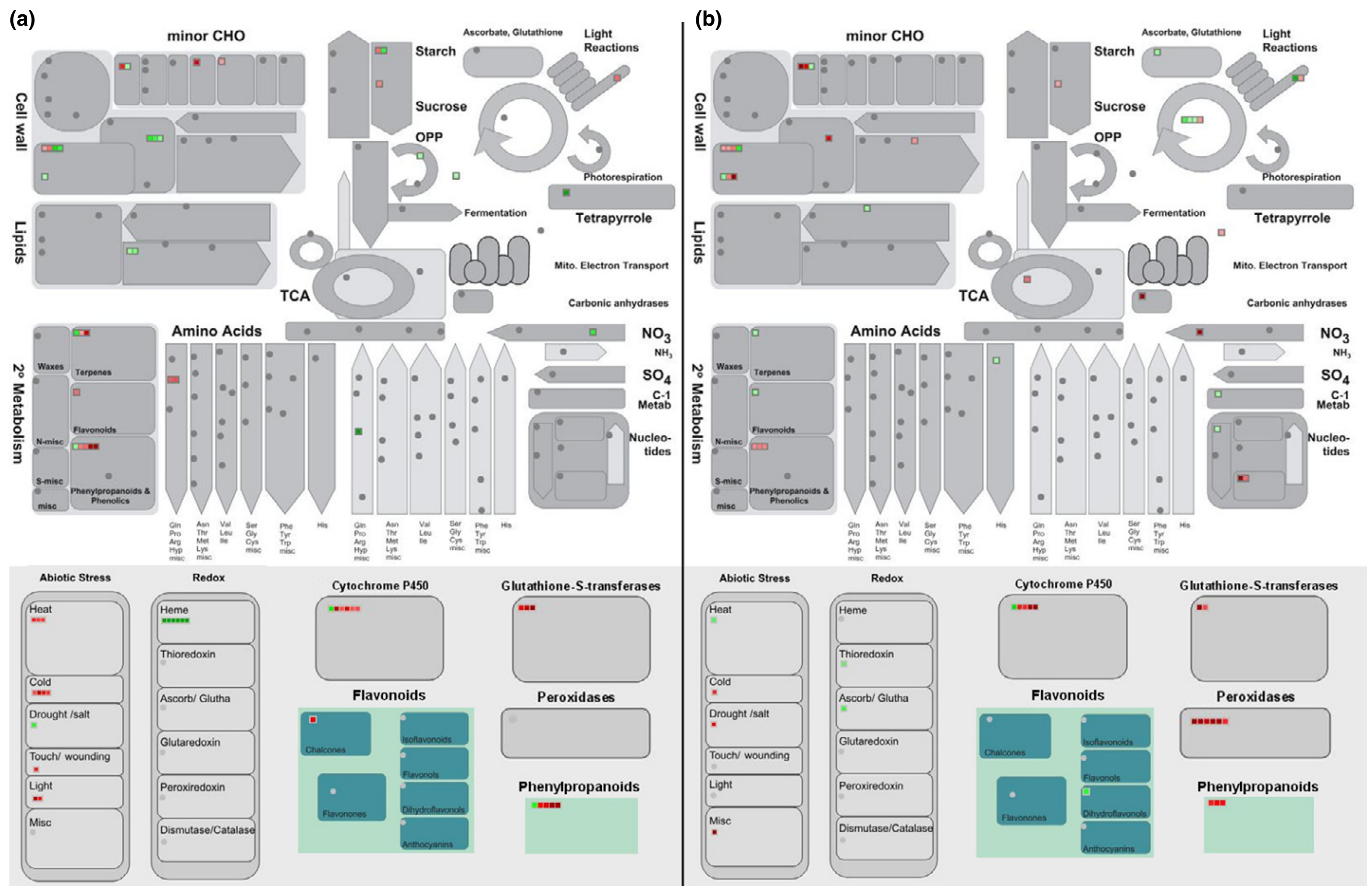


Fig. 5 MapMan analysis of *Medicago truncatula* transcript data of hybridizations 2 (a) and 3 (b). Above, general metabolism overview. Below, abiotic stress and redox response, phenylpropanoid and flavonoid metabolism, and glutathione S-transferase, cytochrome P450 and peroxidase enzyme families. OPP, oxidative pentose phosphate pathway.

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

including the pathogenesis-related proteins 4A and 1 (8.2-fold and 5.4-fold repressed, respectively).

Concerning nodulation, 25% of the down-regulated genes belonged to this category. Only two nodulation genes were up-regulated, chalcone synthase and EST433218, an *M. truncatula* expressed sequence tag (EST) highly induced in roots 24 h after inoculation (VandenBosch *et al.*, 2000). With these exceptions, nodulation genes constituted one of the largest categories of repressed genes, including several leghaemoglobin sequences (from 15.6- to 10.9-fold repressed), *Nodulin 25*, which is expressed in the peribacteroid space (Kiss *et al.*, 1990) (14.3-fold down-regulated), and the nodulin *MtN21* (2.6-fold inhibited).

The MapMan tool revealed that, in general, plant metabolism seemed to be less affected by As(III) when the plant was inoculated in comparison to hybridization 1; in particular, cell wall architecture, photosynthesis and carbohydrate metabolism were less affected (Fig 5a). Expression of genes involved in the response to abiotic stress and in the pathway of phenylpropanoid biosynthesis was enhanced, but flavonoid biosynthesis, which was previously induced in hybridization 1 (Fig. 4b), was no longer activated in inoculated plants. These results suggested that the presence of the bacteria could alleviate the stress produced by arsenite.

Hybridization 3 This third array was designed to analyse the effect of inoculation on the transcriptome of already As-stressed plants. This hybridization can be considered the 'subtraction' of hybridization 2 from hybridization 1. In total, 4509 genes were discarded, 146 genes were up-regulated and 61 genes showed reduced expression (access number E-MTAB-1723; Table S4). Compared with the other two hybridizations, hybridization 3 showed the minimum number of affected genes, suggesting that the symbiotic interaction induced fewer transcriptomic changes compared with As stress. The overall results (Fig. 3c) showed that 15% of the total number of up-regulated genes (26 genes) belonged to the stress response category, most of which were involved in pathogenesis, including the pathogenesis-related protein PR-1 and the pathogen-induced calmodulin-binding protein (3.1- and 2.1-fold induced, respectively). It is known that first stages of the symbiotic interaction share common signalling pathways with pathogenesis (Hentschel *et al.*, 2000; Samac & Graham, 2007). ACC oxidase, which is involved in the ethylene response, was 5.7-fold up-regulated. A GST sequence was 3.1-fold up-regulated. Some members of the GST family were up-regulated in inoculated roots of *M. truncatula* 24 hpi (Lohar *et al.*, 2006).

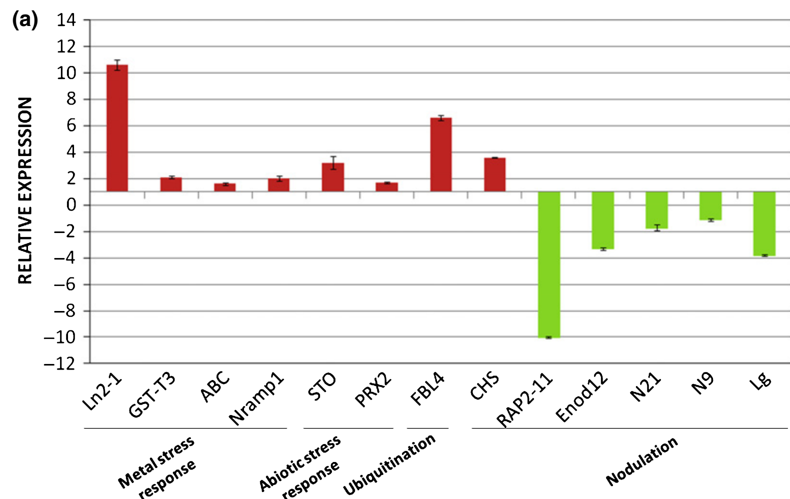
In spite of the presence of As, there was induction of genes related to the early stages of nodulation, including isoflavone-7-O-methyltransferase (flavonoid synthesis) (2.3-fold), LysM (lysin motif) receptor kinase (involved in Nod-factor perception) (17.5-fold), and genes involved in the progression of the infection thread (nodulins *N6*, *MtN15* and *MtN19*) (between 2- and 4-fold). Moreover, E3 ubiquitin-protein ligase (*CERBERUS*), which is involved in the early steps of infection thread formation (Yano *et al.*, 2009), was 5.7-fold overexpressed. These data are similar to those found in transcriptomic analysis of legume nodulation in the absence of arsenite (Mitra *et al.*, 2004; Lohar *et al.*, 2006), demonstrating that plants try to get the symbiotic interaction started even in the presence of As. By contrast, *MtN21*, a transporter induced during nodule development (Gamas *et al.*, 1996), and involved in late stages of nodulation (Hakoyama *et al.*, 2012), was 2.4-fold down-regulated.

Genes in the general metabolism category were strongly repressed in hybridization 3, with 40% of down-regulated genes in this category. Most of the genes involved in the dark and light phases of photosynthesis were inhibited; that is, those encoding chlorophyll a/b-binding protein, the RuBisco small subunit and 6,7-dimethyl-8-ribityllumazine synthase (3.9-fold, 2.9-fold and 2.4-fold, respectively) (Fig. 5). The effects of these genes in root tissues is unknown. MapMan also revealed the up-regulation of several genes involved in the cell wall, for example, a nonspecific lipid-transfer protein (8.6-fold), an expansin (5.1-fold), and a pectin-esterase (5.0-fold). Whether these genes may be involved in infection thread growth is unknown. In contrast to hybridizations 1 and 2, a number of peroxidases were induced, which must be related to nodulation, as both roots compared in hybridization 3 were in the presence of As. It is known that ROS participate in various stages of nodule development, from infection to senescence (Chang *et al.*, 2009).

Validation of the microarray results

The abovementioned results were further confirmed by qRT-PCR. In particular, we focused on nodulation genes and the stress response. Data from qRT-PCR (Fig. 6a) confirmed that the isoforms *Ln2-1* and *GST-T3* of GST were up-regulated 10.5- and 2.1-fold, respectively. Expression of the gene encoding the ABC transporter putatively involved in the transport of the xenobiotic into the vacuole was 1.6-fold enhanced; that of the root-specific metal transporter gene *Nramp1* was increased by 2.0-fold; that of the salt-tolerant protein gene *STO* was 3.2-fold enhanced; and that of the peroxidase gene *PRX2* was 1.7-fold increased. Results showed a strong increase in the expression of *FBL4* (F-box/LRR-repeat protein 4), a gene that encodes a protein of the F-box family containing 19 leucine-rich repeats with ubiquitin-protein ligase activity (Xiao & Jang, 2000). By contrast, the qRT-PCR data confirmed that most of the nodulation genes were repressed, except for chalcone synthase which was 3.8-fold over-expressed, suggesting enhanced synthesis of flavonoids needed for the plant–rhizobia interaction. In fact, a microscopic observation of the root revealed profuse bacterial attachment to the entire root surface in the presence of As(III) (Fig. 6c). However, very few infection threads were observed. In contrast, control roots showed frequent root hair curling and infection thread initiation (Fig. 6b). These results are supported by genetic data, as most of the subsequent nodulation genes were repressed (Fig. 6a): *Enod12* (3.3-fold), *N21* (1.7-fold), *N9* (1.1-fold) and leghaemoglobin (3.8-fold). Moreover, *ERN* (homologous to *Arabidopsis thaliana* AP2/ERF transcription factor *RAP2-11*), which is involved in the ethylene response during nodulation (Penmetsa & Cook, 1997; Middleton *et al.*, 2007),

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust



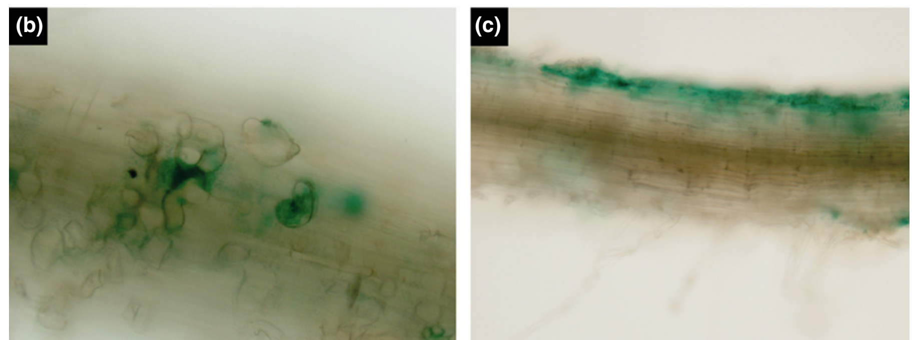


Fig. 6 Real-time RT-PCR confirmation of *Medicago truncatula* microarray data and microscopic observation of inoculated roots in the presence versus the absence of arsenic (As). (a) Expression levels of nodulation and stress response genes in the presence of 25 μM As(III) compared with the absence of metalloid, both in *Ensifer medicae* MA11 inoculated roots. Data are the mean + SE of two independent samples and three replicates of each. Below, microscopic analysis of *M. truncatula* roots with a *lacZ*-labelled strain in the absence (b) and the presence (c) of 25 μM As(III). Ln2-1, glutathione transferase 2-1; GST-T3, glutathione transferase T3; ABC, glutathione ABC transporter; Nramp1, natural resistance-associated macrophage protein 1; STO, Salt Tolerance Protein, PRX2, peroxidase 2; FBL4, F-box/LRR-repeat protein 4; CHS, chalcone synthase; RAP2.11, AP2/ERF transcription factor; Enod12, early nodulin 12; N21, nodulin 21; N9, nodulin 9; Lg, leghaemoglobin.

was 10-fold inhibited (Fig. 6a), and is a putative candidate for genetic engineering.

Meta-analysis of genes modulated by As(III) during the *Medicago truncatula–Ensifer medicae* symbiotic interaction

An analysis by hierarchical clustering was performed to identify genes that were co-regulated by arsenite and rhizobia. Gene expression profiles were compared pairwise and hierarchically clustered arbitrarily into 10 clusters (0–9) containing genes showing similar expression trends (Fig. 7a).

Cluster 0 included 104 genes over-expressed in hybridizations 1 and 2 and showing no significant differences in hybridization 3 (Fig. 7b). Thirty-four per cent of these genes were directly involved in the stress response (*GST*, the peroxidase *P5a*, *Nramp1* and *P5CS*). In general, these genes showed higher expression in hybridization 1 (noninoculated plants) than in hybridization 2 (inoculated plants), again indicating the protection exerted by rhizobia. Five per cent of them were transcription factors, including a homeodomain transcriptional regulator and a *bHLH* (basic helix-loop-helix) transcription factor.

The 388 genes in cluster 1 showed moderate induction in the three hybridizations (Fig. 7c), and were co-regulated by both As and rhizobia. Twenty-seven per cent of the genes were stress-related (*GST*, *STO*, several metal transporters and a P450 cytochrome monooxygenase). The most remarkable finding in this cluster was that 19% of the genes were transcription factor genes belonging to the *BZIP* (Basic Leucine Zipper domain), *MYB* (Myeloblast DNA-Binding Domain) and *bHLH* (basic helix-loop-helix domain) families.

Cluster 2 included 161 genes which showed no significant changes in hybridizations 1 and 2 and moderate over-expression in hybridization 3, suggesting that they responded more to inoculation than to the presence of As. Hence, they were not further considered.

Cluster 3 included 332 genes showing moderate induction in hybridizations 2 and 3, without significant differences in hybridization 1 (Fig. 7d). This cluster contained genes induced in the presence of the microsymbiont (chalcone synthase, chalcone reductase, the pathogenesis-related protein (PRP) gene *PRP4*, *ENODL8* and *ENOD18*). Three per cent of the genes encoded transcription factors.

Cluster 4 contained 565 genes showing minor overexpression in the three hybridizations which were not further considered.

Cluster 5 included 571 genes showing slight induction in hybridization 1 and nonsignificant changes in hybridizations 2 and 3. These genes, related to stress independently of the presence of bacteria, were not further investigated.

Cluster 6 contained 187 genes with moderate inhibition in the three hybridizations (Fig. 7e). These genes were negatively co-regulated by As and rhizobia. In this cluster, 23% of the genes were related to the cell wall, including cellulose synthase and xyloglucan endotransglucosilase/hydrolase. The early nodulins

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

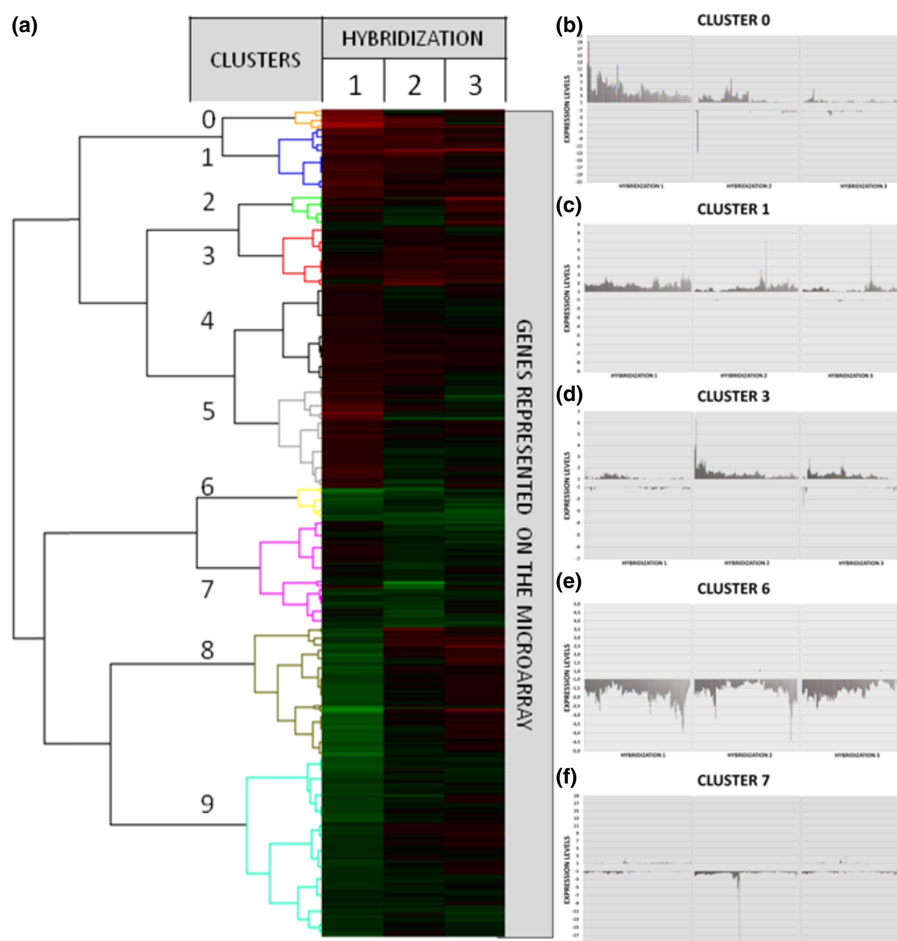


Fig. 7 Pairwise comparison and hierarchical clustering of *Medicago truncatula* gene profiles of the three hybridizations. (a) Genes were arbitrarily grouped into 10 clusters. (b–f) Expression levels of the genes included in clusters 0, 1, 3, 6 and 7, respectively.

MtN9 and *ENOD12*, which are involved in infection thread progression, belonged to this cluster. In addition, two nodule-specific cysteine-rich peptides, 111 and 319, putatively involved in nodule morphogenesis (Van de Velde *et al.*, 2010), were found. Notably, the nitrate transporter at the plasma membrane was inhibited both by As and in inoculated plants.

Cluster 7 contained 600 genes showing nonsignificant differences in hybridizations 1 and 3, and repression in hybridization 2 (Fig. 7f), and included genes affected by As only in inoculated plants. In addition to the nitrate transporter (cluster 6), nitrite reductase and ferredoxin-NADP reductase were repressed, indicating inhibition of the nitrate assimilatory pathway. The two most important groups were nodulation genes and transcription factors. Nodulation genes included *MtN25* (expressed in the peribacteroid space), *MtN21* (a nodule-specific transporter), *MtENOD16* (involved in signal transduction during nodulation), *MtN29* (with unknown function), *MtEnod8.1* (involved in lipid metabolism during nodulation), leghaemoglobin and several cysteine-rich peptides involved in nodule morphogenesis. This category included several transcription factors (of the zinc finger, *WRKY* (Worky DNA binding proteins) and *RAV* (Related to ABI3/VP1 transcription factors)-like families) and the ethylene-responsive transcription factor *ERN*.

Cluster 8 contained 727 genes (showing weak inhibition in hybridization 1) which were related to the stress response, independently of the presence of bacteria, and were not further considered.

Cluster 9 contained 1010 genes showing minor inhibition in the three hybridizations.

Identification of genes playing central roles in stress and nodulation

Venn diagrams were generated from the results for the three arrays, in order to identify genes that were jointly induced (Fig. 8a) or repressed (Fig. 8b). Comparison of hybridizations 1 and 2 showed 32 genes that were jointly up-regulated, 12 of them related to stress (e.g. *GST*, *VuP5CS, STOARATH* and an ABC transporter protein). In contrast, 10 genes were jointly down-regulated, four of which are involved in cell wall synthesis (extensin-like protein, cellulose synthase and pectin esterase). After comparison of hybridizations 1 and 3, results showed 10 jointly up-regulated genes (including three transcription factor genes of the *bHLH* and *Myb* families) and four jointly repressed genes (including a gene encoding RuBisCo and a gene with homology to *At1g54780*, which is involved in photosystem II repair). Comparison of hybridizations 2 and 3 showed 16 up-regulated genes (*GST*, a cytochrome P450 and two transcription factor genes belonging to the *bHLH* and *Myb* families) and seven repressed genes, including the protein UPF0603 (involved

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

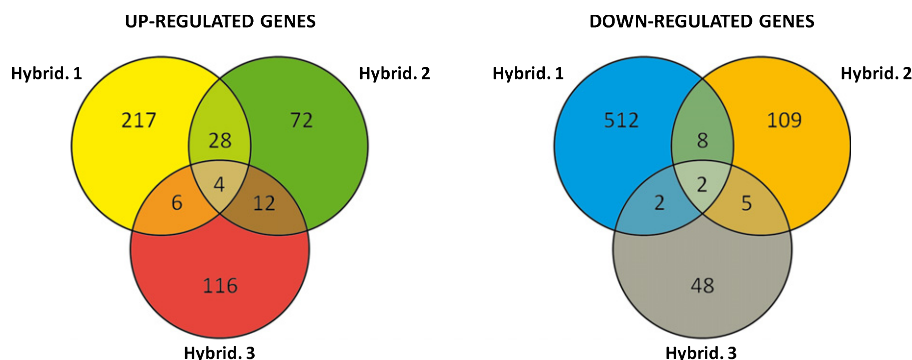


Fig. 8 Comparison by Venn diagram of genes jointly up-regulated or down-regulated by As(III) and rhizobia in *Medicago truncatula*. The intersections in the Venn diagram show genes up- or down-regulated in two or three hybridizations simultaneously.

Table 3 *Medicago truncatula* genes jointly induced or repressed in the three hybridizations, which occupy the central position of Venn diagrams

		Gene	Description	Reference
Up-regulated	Consensus sequence	Basic helix-loop-helix dimerization domain (bHLH)	Superfamily of transcription factors that bind, as dimers, to specific DNA target sites. Important regulatory components in diverse plant biological processes	Toledo-Ortiz *et al.* (2003)
	(TC91910)	Putative wound-induced protein (*Medicago sativa*) (97% identity)	Sequence described as a molecular marker for organogenetic pathways acting in alfalfa (*Medicago sativa*) nodule development	Jiménez-Zurdo *et al.* (2000)
	Consensus sequence	Myb-DNA binding domain	Superfamily of transcription factors involved in cell cycle, the identity and fate of plant cells, and secondary metabolism	Stracke *et al.* (2001)
	(TC85431)	Nonspecific lipid-transfer protein precursor (100% identity to chickpea (*Cicer arietinum*) NLTPCICAR)	Family of small cysteine-rich lipid-binding proteins that play a key role in plant resistance to biotic and abiotic stress, in particular, in plant defence mechanisms against phytopathogenic bacteria and fungi and, possibly, in the assembly of hydrophobic protective layers of surface polymers. Many of them act as potent allergens	Breiteneder & Mills (2005); Salcedo *et al.* (2007)
Down-regulated	(TC76733)	Pectinesterase	Ubiquitous cell-wall-associated enzyme that presents several isoforms that act in the modification of cell walls via demethylesterification of cell wall pectin	Micheli (2001)
	(TC85286)	18.3-kDa thylakoid lumen protein	Chloroplast protein involved in photosystem II repair. The function of this protein in roots is unknown	Sirpiö *et al.* (2007)

in photosystem II repair) and the nodulin *MtN21*, a vacuolar membrane iron transporter needed for the symbiosome and/or bacteroid differentiation (Hakoyama *et al.*, 2012).

Only four genes (Table 3) were up-regulated simultaneously in the three hybridizations, including two transcriptions factor genes of the *MYB* and *bHLH* families, and a member of the nonspecific lipid-transfer protein (nsLTP) superfamily. A member of this superfamily (*MtN5*) is implicated in the epidermal stages of the *Rhizobium*–host interaction (Pii *et al.*, 2012). In the same context, a wound-induced protein (TC91910) located at the central position of the Venn diagram was described as a molecular marker for nodule organogenesis (Jiménez-Zurdo *et al.*, 2000). Only two genes were jointly down-regulated in the three hybridizations (Table 3), which could occupy a central role in the response of the symbiotic interaction to As(III).

Overexpression of selected genes in composite plants

In order to assess the functions of some of the selected genes, composite plants, in which only the root part is transgenic upon infection with *A. rhizogenes* (Boisson-Dernier *et al.*, 2001), were generated. This tool is widely used for demonstration of gene function in *M. truncatula*, especially for processes occurring in the roots (Cerri *et al.*, 2012; Zélicourt *et al.*, 2012). Two genes were selected in order to improve nodulation, reduce root stress, or both: the ethylene-responsive transcription factor gene related to nodulation *ERN* (Middleton *et al.*, 2007; Cerri *et al.*, 2012), belonging to cluster 7 and the early nodulin *ENOD12*, belonging to cluster 3. Composite plants expressing *ERN* (Fig. 9) showed no statistically significant differences in shoot parameters (biomass and number of trifolii) as compared with control

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

plants (transformed with the empty vector). Significant differences were recorded in root length (a parameter related to As toxicity), as the expression of *ERN* was able to prevent root growth inhibition caused by As toxicity (Figs 9c, 10). An increase of 3-fold in the number of nodules formed in the presence of As was found (Figs 9e, 10), although there was no significant difference from the control because of the high heterogeneity of transgenic roots.

Composite plants expressing *ENOD12* (Fig. 9) showed no statistically significant differences in shoot parameters compared with control plants, or in root length (Figs 9c, 10). In this case, the expression of the early nodulin *ENOD12* in roots led to a 2-fold increase in the number of nodules formed in the absence of As and a 10-fold increase in the number of nodules formed in the presence of As, the differences being significant at $P < 0.05$ (Figs 9e, 10).

Discussion

The physiology of the effect of As on plants has been widely studied, in relation to inhibition of seed germination and root growth and reduction of the photosynthetic rate (Rahman *et al.*, 2007; Mateos-Naranjo *et al.*, 2012). Recently, the term 'arsenomics' has been proposed as the approach dealing with transcriptome, proteome, and metabolome alterations during plant adaptation to As (Tripathi *et al.*, 2012b). However, most of these results concern nonlegume plants (Abercrombie *et al.*, 2008; Chakrabarty *et al.*, 2009; Duquesnoy *et al.*, 2009; Srivastava *et al.*, 2009; Rao *et al.*, 2011; Finnegan & Chen, 2012; Tripathi *et al.*, 2012a), and the effect of As on the symbiotic interaction has not been examined from a global perspective. By contrast, several transcriptomic approaches focus on the legume–rhizobia interaction, independently of any stress (Fedorova *et al.*, 2002; Küster *et al.*, 2007; Brechenmacher *et al.*, 2008; Hernández *et al.*, 2009; Moreau *et al.*, 2011). In this work, split-roots, microscopy, transcriptomic meta-analysis and composite plants were used, in order to unravel the effect of As on the symbiosis.

The results for split-roots suggested a local (nonsystemic) effect of As(III) on both nodulation and root elongation. The first approach in the meta-analysis was the global evaluation of the effect of arsenite on the plant, independently of nodulation. This is the first transcriptomic analysis of this type in a legume, and the results are consistent with those previously reported for other plants, showing activation of the abiotic stress response (cold, heat shock, drought and salt tolerance). To cope with oxidative stress generated by As (Leterrier *et al.*, 2012; Tripathi *et al.*,

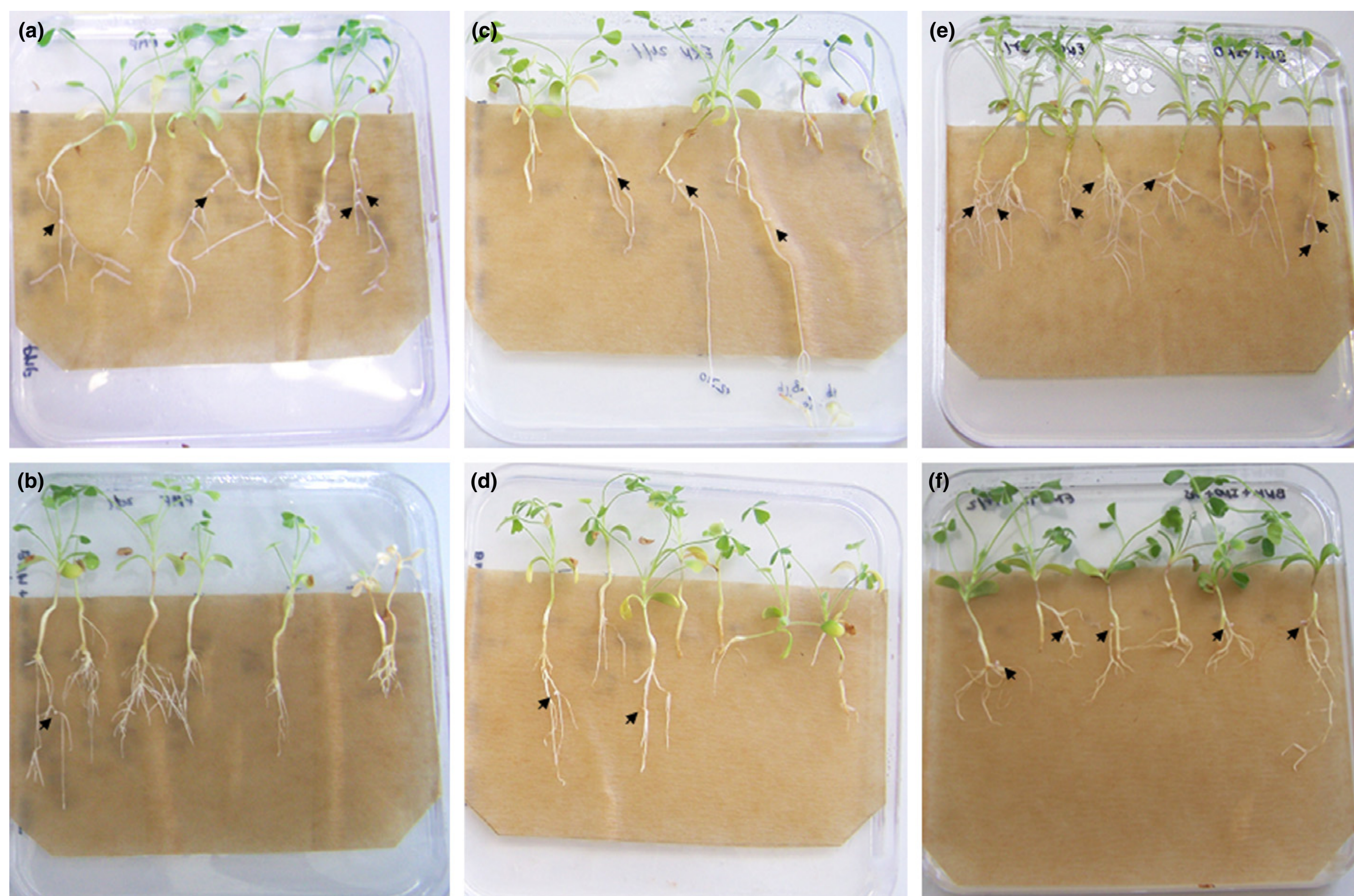


Fig. 9 Expression of *ERN* and *ENOD12* in composite *Medicago truncatula* plants. Composite plants expressing *ERN* (ethylene responsive factor) (c, d) or *ENOD12* (early nodulin 12) (e, f) were generated, together with control plants with the empty vector (a, b). Plants were cultivated in the absence (a, c, e) or the presence (b, d, f) of arsenic (As) and inoculated with *Ensifer medicae* MA11. Plants were photographed at 28 dpi (days post-inoculation). Arrowheads indicate the position of nodules.

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

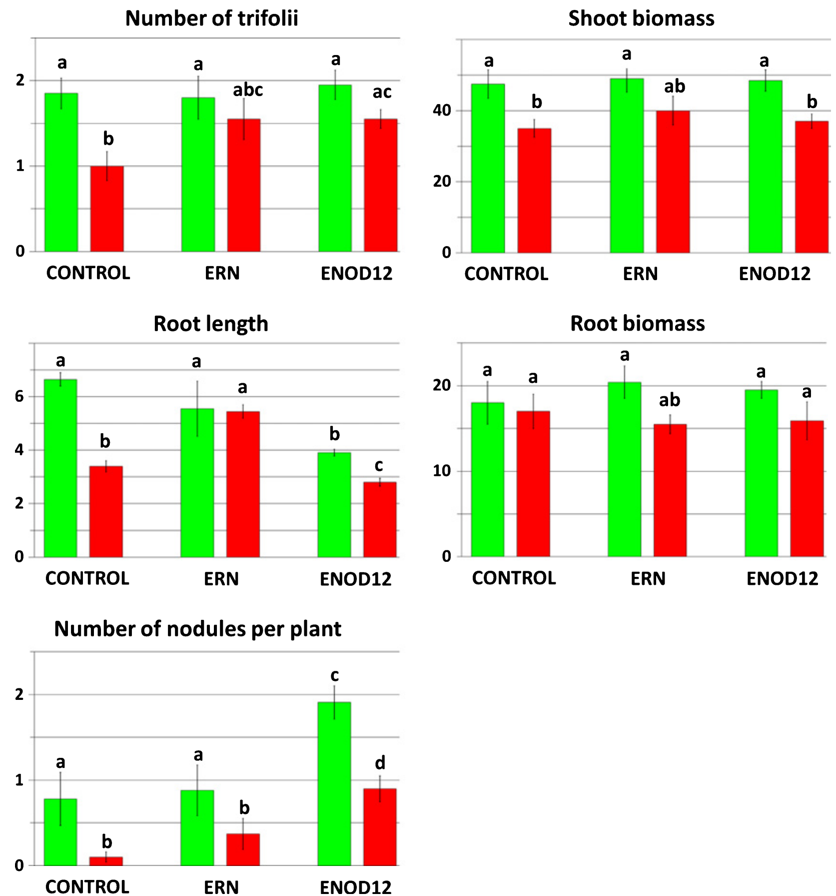


Fig. 10 Growth parameters and nodulation of *Medicago truncatula* composite plants expressing the ethylene responsive factor (*ERN*) or the early nodulin12 (*ENOD12*). The number of trifolii, shoot and root biomass, root length and number of nodules per plant were determined in inoculated *M. truncatula* composite plants at 28 d post-inoculation (dpi) in the presence (red bars) and the absence (green bars) of arsenic (As). Data are mean ± SE of 19–25 plants. Significant differences at $P < 0.05$ are indicated by different letters.

2012a), plants have evolved a complex antioxidative defence network that comprises enzymatic (peroxidase, catalase, superoxide dismutase, glutathione reductase, etc.) and nonenzymatic (glutathione, ascorbate, proline, tocopherol, carotenes, etc.) mechanisms (Sharma & Dietz, 2009; Sharma *et al.*, 2012). Phenolic compounds also play an important role in the detoxification of free radicals (Ksouri *et al.*, 2007).

In our study, glutathione was found to play a central role in both As tolerance and detoxification, as previously reported (Ahsan *et al.*, 2008; Tripathi *et al.*, 2012b). GSH has redox buffering capacity and, moreover, As(III) forms stable complexes with glutathione and phytochelatins (PCs) (Raab *et al.*, 2004), which are transported inside the vacuoles of root cells by ABCC transporters at the tonoplast (Song *et al.*, 2010). This mechanism is strongly dependent on S supply (Rai *et al.*, 2011; Tuli, 2011; Reid *et al.*, 2013); thus, genes of the S assimilation pathway were overexpressed. Moreover, secondary metabolism was strongly enhanced, especially the synthesis of (iso)flavonoids and phenylpropanoids, molecules that play important roles as ROS scavengers in plants (Pietta, 2000; Winkel-Shirley, 2001). In contrast to data for rice and maize (Requejo & Tena, 2005; Tripathi *et al.*, 2012a), antioxidant enzymes were not activated, but repressed or unaffected. Our results may imply that, at the concentrations of As(III) used, the main oxidative response in *M. truncatula* is via aromatic compounds or that As(III) induces different stress signalling compared with As(V), as has previously been reported (Srivastava *et al.*, 2007; Chakrabarty *et al.*, 2009). In the context of a legume plant, the additional implication of flavonoids in plant–rhizobia signalling must be also considered, and could be envisioned as the plant tendency to establish symbiosis face to As exposure.

The second approach was the comparison of the transcriptomic profiles of As- exposed versus unexposed plants, both inoculated. Two remarkable conclusions were obtained; the first was that inoculated plants showed a reduction in the stress response. Many previous studies reported that inoculation with resistant rhizobia protected plant roots from the deleterious effects of metals (Reichman, 2007; Wani *et al.*, 2008; Dary *et al.*, 2010), but, to our knowledge, this is the first molecular confirmation of the protective effect of As-tolerant rhizobia. Rhizosphere bacteria alleviate metal stress by several mechanisms, such as nitrogen fixation, phosphate solubilization, synthesis of siderophores, production of auxins, and ACC-deaminase activity (Glick, 2010; Glick, 2012; Ahemad & Kibret, 2014; De Bashan *et al.*, 2012). Furthermore, the presence of rhizobia decreases metal accumulation in plants (Reichman, 2007; Wani *et al.*, 2008; Dary *et al.*, 2010), as rhizobia may restrict As from moving into the root by adsorbing the metalloid onto the bacterial cell surface (Abd-Alla *et al.*, 2012). In addition to this passive mechanism, bacteria have evolved several mechanisms for As detoxification, including complexation, extrusion from the bacterial cell, oxidation/reduction, and methylation (Bhattacharjee & Rosen, 2007). The second conclusion, based on the extreme induction of chalcone synthase, was that the plant is biased towards establishing interactions with rhizobia

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

under stress conditions. High concentrations of flavonoids, which are the first molecules involved in the plant–rhizobia crosstalk, could be the reason for the extended rhizobial colonization of the root at 5 dpi. However, the inhibition of subsequent nodulation genes (involved in Nod factor perception, infection thread initiation and progression, and nodule organogenesis) leads to abortion of nodulation, as previously reported (Pajuelo *et al.*, 2008).

The third hybridization, comparing inoculated versus noninoculated roots, both in the presence of As, showed a relatively low number of affected genes, indicating that nodulation induces fewer transcriptomic changes compared with As stress. A high proportion of the over-expressed genes were genes involved in pathogenesis, confirming the known relationship between pathogenesis and nodulation at the first stages of the symbiosis (Soto *et al.*, 2009).

Hierarchical clustering grouped genes with similar regulation. Clusters 0, 1 and 3 grouped genes more or less up-regulated in the three hybridizations, widely represented by genes involved in stress response. Moreover, clusters 6 and 7 grouped genes that were repressed in the three hybridizations. Notably, 13 nodulation genes fell into these two clusters, indicating the high repression of the nodulation process, with the exception of chalcone synthase.

Finally, Venn diagrams were used in order to look for target genes that could occupy a central role in both processes. Transcription factors belonging to the Myb and bHLH families were located in this central position. Some of the members of these families are involved in metal homeostasis or nodulation (Van de Mortel *et al.*, 2008; Godiard *et al.*, 2011; Ariel *et al.*, 2012; Borges-Osorio *et al.*, 2012).

In order to confirm our results, composite plants expressing a transcription factor (*ERN*) and an early nodulin (*ENOD12*) were generated. The results showed that *ERN* was involved both in improving nodulation and in alleviating root growth inhibition. By contrast, the overexpression of *ENOD12* greatly enhanced nodulation in the presence of As (up to 10-fold), but did not have any effect on root elongation in the presence of the toxin.

Legumes accumulate As (and metals) mainly in the roots (Pajuelo *et al.*, 2007, 2011; Reichman, 2007; El Aafi *et al.*, 2012), and this is adequate for metal phytostabilization (Vázquez *et al.*, 2006; Dary *et al.*, 2010; El Aafi *et al.*, 2012), as it immobilizes metals in the rhizosphere of the plants without increasing translocation to shoots (Méndez & Maier, 2008). In particular, autochthonous legumes and resistant rhizobia from metal-polluted soils are the most effective partnerships (Maynaud *et al.*, 2013). However, for this technique to be useful, plant establishment in polluted soils must be increased. Inoculation with resistant rhizobia could be a way to improve plant performance under stress conditions (Figueiredo *et al.*, 2008; Pajuelo *et al.*, 2011; Zaidi *et al.*, 2012) and usually decreases metal content in plants (Dary *et al.*, 2010; El Aafi *et al.*, 2012). In addition to inoculation with appropriate symbiotic partners, genetic engineering can improve nodulation and/or reduce stress in legumes. In addition to *ERN* and *ENOD12*, the newly identified transcription factors – in view of their amplifying effect – may constitute appropriate additional targets for biotechnological approaches with the aim of improving tolerance (but not accumulation) and nodulation under As stress.

Acknowledgements

This work was financed by MINECO (Spain)/FEDER (BIO-2009-7766) and Junta de Andalucia (P11-RNM-7274). A.L. acknowledges a grant from Junta de Andalucía. The authors thank Mrs Asunción Romero for technical assistance.

References

Abd-Alla MH, Morsy FM, El-Enany AWE, Ohyama T. 2012. Isolation and characterization of a heavy-metal-resistant isolate of *Rhizobium leguminosarum* bv. *viciae* potentially applicable for biosorption of Cd^{2+} and Co^{2+}. *International Biodeterioration & Biodegradation* **67**: 48–55.

Abercrombie JM, Halfhill MD, Ranjan P, Rao MR, Saxton AM, Yuan JS, Stewart CN Jr. 2008. Transcriptional responses of *Arabidopsis thaliana* plants to As(V) stress. *BMC Plant Biology* **8**: 87–96.

Adriano DC. 2001. *Trace elements in terrestrial environments: biogeochemistry, bioavailability and risks of metals*, 2nd edn. New York, NY, USA: Springer.

Ahemad M, Kibret M. 2014. Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. *Journal of King Saud University-Science* **26**: 1–20.

Ahsan N, Lee DG, Alam I, Kim PJ, Lee JJ, Ahn YO, Kwak SS, Lee IJ, Bahk JD, Kang KY *et al.* 2008. Comparative proteomic study of arsenic-induced differentially expressed proteins in rice roots reveals glutathione plays a central role during As stress. *Proteomics* **8**: 3561–3576.

Ariel F, Brault-Hernandez M, Laffont C, Huault E, Brault M, Plet J, Moison M, Blanchet S, Ichanté JL, Chabaud M *et al.* 2012. Two direct targets of cytokinin signalling regulate symbiotic nodulation in *Medicago truncatula*. *Plant Cell* **24**: 3838–3852.

Bhattacharjee H, Rosen BP. 2007. Arsenic metabolism in Prokaryotic and Eukaryotic Microbes. In: Nie DH, Silver S, eds. *Molecular microbiology of heavy metals*. Berlin-Heidelberg, Germany: Springer-Verlag, 371–406.

Bienert GP, Thorsen M, Schüssler MD, Nilsson HR, Wagner A, Tamás MJ, Jahn TP. 2008. A subgroup of plant aquaporins facilitate the bi-directional diffusion of $As(OH)_3$ and $Sb(OH)_3$ across membranes. *BMC Biology* **6**: 26.

Boisson-Dernier A, Chabaud M, Garcia F, Becard G, Rosenberg C, Barker DG. 2001. *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Molecular Plant-Microbe Interactions* **14**: 695–700.

Borges-Osorio M, Bücker-Neto L, Castilhos G, Turchetto-Zolet AC, Wiebke-Strohm B, Bodanese-Zanettini MH, Margis-Pinheiro M. 2012. Identification and *in silico* characterization of soybean trihelix-GT and bHLH transcription factors involved in stress responses. *Genetics and Molecular Biology* **35**(1 Suppl): 233–246.

Brechenmacher L, Kim MY, Benitez M, Li M, Joshi T, Calla B, Lee MP, Libault M, Vodkin LO, Xu D *et al.* 2008. Transcription profiling of soybean nodulation by *Bradyrhizobium japonicum*. *Molecular Plant-Microbe Interactions* **21**: 631–645.

Breiteneder H, Mills C. 2005. Nonspecific lipid-transfer proteins in plant foods and pollens: an important allergen class. *Current opinion in Allergy and Clinical Immunology* **5**: 275–279.

Briat JF. 2010. Arsenic tolerance in plants: "Pas de deux" between phytochelatin synthesis and ABCC vacuolar transporters. *Proceedings of the National Academy of Sciences, USA* **107**: 20853–20854.

Broos K, Beyens H, Smolders E. 2005. Survival of rhizobia in soil is sensitive to elevated zinc in the absence of the host plant. *Soil Biology & Biochemistry* **37**: 573–579.

Cerri MR, Frances L, Laloum T, Auriac MC, Niebel A, Oldroyd GED, Barker DG, Fournier J, de Carvalho-Niebel F. 2012. *Medicago truncatula* ERN transcription factors: regulatory interplay with NSP1/NSP2 GRAS factors and

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

expression dynamics throughout rhizobial infection. *Plant Physiology* **160**: 2155–2172.

Chakrabarty D, Trivedi PK, Misra P, Tiwari M, Shri M, Shukla D, Kumar S, Rai A, Pandey A, Nigam D *et al.* 2009. Comparative transcriptome analysis of arsenate and arsenite stresses in rice seedlings. *Chemosphere* 74: 688–702.

Chang C, Damiani I, Puppo A, Frendo P. 2009. Redox changes during the legume–*Rhizobium* symbiosis. *Molecular Plant* **2**: 370–377.

Dary M, Chamber-Pérez MA, Palomares AJ, Pajuelo E. 2010. '*In situ*' phytostabilisation of heavy metal polluted soils using *Lupinus luteus* inoculated with metal resistant plant-growth promoting rhizobacteria. *Journal of Hazardous Materials* **177**: 323–330.

De Bashan LE, Hernández JP, Bashan Y. 2012. The potential contribution of plant growth promoting bacteria to reduce environmental degradation – a comprehensive evaluation. *Applied Soil Ecology* **61**: 171–189.

Dondrup M, Goesmann A, Bartels D, Kalinowski J, Krause L, Linke B, Rupp O, Sczyrba A, Pühler A, Meyer F. 2003. EMMA: a platform for consistent storage and efficient analysis of microarray data. *Journal of Biotechnology* **106**: 135–146.

Duan GL, Zhou Y, Tong YP, Mukhopadhyay R, Rosen BP, Zhu YG. 2007. A CDC25 homologue from rice functions as an arsenate reductase. *New Phytologist* **174**: 311–321.

Duker AA, Carranza EJM, Hale M. 2005. Arsenic geochemistry and health. *Environment International* **31**: 631–641.

Duquesnoy I, Goupil P, Nadaud I, Branlard G, Piquet-Pissaloux A, Ledoigt G. 2009. Identification of *Agrostis tenuis* leaf proteins in response to As(V) and As (III) induced stress using a proteomics approach. *Plant Science* **176**: 206–213.

Ehrhardt DW, Atkinson EM, Long SR. 1992. Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. *Science* **256**: 998–1000.

El Aafi N, Brhada F, Dary M, Filali-Maltouf A, Pajuelo E. 2012. Rhizostabilization of metals in soils using *Lupinus luteus* inoculated with the metal resistant rhizobacterium *Serratia* sp. MSMC541. *International Journal of Phytoremediation* **14**: 261–274.

Ellis DR, Gumaelius L, Indriolo E, Pickering IJ, Banks JA, Salt DE. 2006. A novel arsenate reductase from the arsenic hyperaccumulating fern *Pteris vittata*. *Plant Physiology* **141**: 1544–1554.

Fahraeus G. 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *Journal of General Microbiology* **16**: 374–381.

Fedorova M, van de Mortel J, Matsumoto PA, Cho J, Town CD, VandenBosch KA, Gantt JS, Vance CP. 2002. Genome-wide identification of nodule-specific transcripts in the model legume *Medicago truncatula*. *Plant Physiology* **130**: 519–537.

Figueiredo M, Burity H, Martínez C, Chanway C. 2008. Alleviation of drought stress in the common bean (*Phaseolus vulgaris* L.) by co-inoculation with *Paenibacillus polymyxa* and *Rhizobium tropici*. *Applied Soil Ecology* **40**: 182–188.

Finnegan PM, Chen W. 2012. Arsenic toxicity: the effects on plant metabolism. *Frontiers in Physiology* **3**: 182.

Gallardo K, Firnhaber C, Zuber H, Hericher D, Belghazi M, Henry C, Küster H, Thompson RD. 2007. A combined proteome and transcriptome analysis of developing *Medicago truncatula* seeds. *Molecular and Cellular Proteomics* **6**: 2165–2179.

Gamas P, Carvalho NF, Lescure N, Cullimore JV. 1996. Use of a subtractive hybridization approach to identify new *Medicago truncatula* genes induced during root nodule development. *Molecular Plant-Microbe Interactions* **9**: 233–242.

Garg N, Singla P. 2011. Arsenic toxicity in crop plants: physiological effects and tolerance mechanisms. *Environmental Chemistry Letters* **9**: 303–321.

Ghosh M, Shen J, Rosen BP. 1999. Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences, USA* **96**: 5001–5006.

Glick BR. 2010. Using soil bacteria to facilitate phytoremediation. *Biotechnology Advances* **28**: 367–374.

Glick BR. 2012. Plant growth promoting bacteria: mechanisms and applications. *Scientifica* **2012**: article ID 963401.

Godiard L, Lepage A, Moreau S, Laporte D, Verdenaud M, Timmers T, Gamas P. 2011. MtbHLH1, a bHLH transcription factor involved in *Medicago truncatula* nodule vascular patterning and nodule to plant metabolic exchanges. *New Phytologist* **191**: 391–404.

Goffard N, Weiller G. 2006. Extending MapMan: application to legume genome arrays. *Bioinformatics* **22**: 2958–2959.

Hakoyama T, Niimi K, Yamamoto T, Isobe S, Sato S, Nakamura Y, Tabata S, Kumagai H, Umehara Y, Brossuleit K *et al.* 2012. The integral membrane protein SEN1 is required for symbiotic nitrogen fixation in *Lotus japonicus* nodules. *Plant and Cell Physiology* **53**: 225–236.

Harborne JB. 1984. *Phytochemical methods: a guide to modern techniques of plant analysis*. London, UK: Chapman and Hall.

Hentschel U, Steinert M, Hacker J. 2000. Common molecular mechanisms of symbiosis and pathogenesis. *Trends in Microbiology* **8**: 226–231.

Hernández G, Valdés-López O, Ramírez M, Goffard N, Weiller G, Aparicio-Fabre R, Fuentes SI, Erban A, Kopka J, Udvardi MK *et al.* 2009. Global changes in the transcript and metabolic profiles during symbiotic nitrogen fixation in phosphorus-stressed common bean plants. *Plant Physiology* **151**: 1221–1238.

Hohnjec N, Vieweg ME, Puhler A, Becker A, Kuster H. 2005. Overlaps in the transcriptional profiles of *Medicago truncatula* roots inoculated with two different *Glomus* fungi provide insights into the genetic program activated during arbuscular mycorrhiza. *Plant Physiology* **137**: 1283–1301.

Huang TL, Nguyen QTT, Fu SF, Lin CY, Chen YC, Huang HJ. 2012. Transcriptomic changes and signalling pathways induced by arsenic stress in rice roots. *Plant Molecular Biology* **80**: 587–608.

Isayenkov SV, Maathuis FJM. 2008. The *Arabidopsis thaliana* aquaglyceroporin AtNIP7:1 is a pathway for arsenite uptake. *FEBS Letters* **582**: 1625–1628.

Jiménez-Zurdo JI, Frugier F, Crespi MD, Kondorosi A. 2000. Expression profiles of 22 novel molecular markers for organogenetic pathways acting in alfalfa nodule development. *Molecular Plant-Microbe Interactions* **13**: 96–106.

Kiss GB, Vincze E, Végh Z, Toth G, Soos J. 1990. Identification and cDNA cloning of a new nodule-specific gene, Nms-25 (nodulin-25) of *Medicago sativa*. *Plant Molecular Biology* **14**: 467–475.

Krzesłowska M. 2011. The cell wall in plant cell response to trace metals: polysaccharide remodeling and its role in defence strategy. *Acta Physiologiae Plantarum* **33**: 35–51.

Ksouri R, Megdiche W, Debez A, Falleh H, Grignon C, Addelly C. 2007. Salinity effects on polyphenol content and antioxidant activities in leaves of de halophyte *Cakile maritime*. *Plant Physiology and Biochemistry* **45**: 244–249.

Küster H, Becker A, Firnhaber C, Hohnjec N, Manthey K, Perlick AM, Bekel T, Dondrup M, Henckel K, Goesmann A *et al.* 2007. Development of bioinformatic tools to support EST-sequencing, *in silico*- and microarray-based transcriptome profiling in mycorrhizal symbioses. *Phytochemistry* **68**: 19–32.

Küster H, Hohnjec N, Krajinski F, El YF, Manthey K, Gouzy J, Dondrup M, Meyer F, Kalinowski J, Brechenmacher L *et al.* 2004. Construction and validation of cDNA-based Mt6k-RIT macro- and microarrays to explore root endosymbioses in the model legume *Medicago truncatula*. *Journal of Biotechnology* **108**: 95–113.

Lafuente A, Pajuelo E, Caviedes MA, Rodríguez-Llorente ID. 2010. Reduced nodulation in alfalfa induced by arsenic correlates with altered expression of early nodulins. *Journal of Plant Physiology* **167**: 286–291.

Lakzian A, Murphy P, Turner A, Beynon JL, Giller KE. 2002. *Rhizobium leguminosarum* bv. *viciae* population in soils with increasing heavy metal contamination: abundance plasmid profiles, diversity and metal tolerance. *Soil Biology & Biochemistry* **34**: 519–529.

Leterrier M, Airaki M, Palma JM, Chaki M, Barroso JB, Corpas FJ. 2012. Arsenic triggers the nitric oxide (NO) and S-nitrosoglutathinone (GSNO) metabolism in *Arabidopsis*. *Environmental Pollution* **166**: 136–143.

Liu WJ, Wood BA, Raab A, McGrath SP, Zhao FJ, Feldmann J. 2010. Complexation of arsenite with phytochelatins reduces arsenite efflux and translocation from roots to shoots in *Arabidopsis*. *Plant Physiology* **152**: 2211–2221.

Lohar DP, Sharopova N, Endre G, Penuela S, Samac D, Town C, Silverstein KAT, VandenBosch KA. 2006. Transcript analysis of early nodulation events in *Medicago truncatula*. *Plant Physiology* **140**: 221–234.

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

Lomax C, Liu WJ, Wu L, Xue K, Xiong J, Zhou J, McGrath SP, Meharg AA, Miller AJ, Zhao FJ. 2012. Methylated arsenic species in plants originate from soil microorganisms. *New Phytologist* **19**: 665–672.

Ma JF, Tamai K, Yamaji N, Mitani N, Konishi S, Katsuhara M, Ishiguro M, Murata Y, Yano M. 2006. A silicon transporter in rice. *Nature* **440**: 688–691.

Ma JF, Yamaji N, Mitani N, Xu XY, Su YH, McGrath SP, Zhao FJ. 2008. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. *Proceedings of the National Academy of Sciences, USA* **105**: 9931–9935.

Mascher R, Lippmann B, Holzinger S, Bergmann H. 2002. Arsenate toxicity: effects on oxidative stress response molecules and enzymes in red clover plants. *Plant Science* **163**: 961–969.

Mateos-Naranjo E, Andrades-Moreno L, Redondo Gómez S. 2012. Tolerance to and accumulation of arsenic in the cordgrass *Spartina densiflora* Brongn. *Bioresource Technology* **104**: 187–194.

Maynaud G, Brunel B, Mornico D, Durot M, Severac D, Dubois E, Navarro E, Cleyet-Marel JC, Le Quéré A. 2013. Genome-wide transcriptional responses of two metal-tolerant symbiotic *Mesorhizobium* isolates to Zinc and Cadmium exposure. *BMC Genomics* **14**: 292.

Meharg AA, Jardine L. 2003. Arsenite transport into paddy rice (*Oryza sativa*) roots. *New Phytologist* **157**: 39–44.

Méndez MO, Maier RM. 2008. Phytostabilisation of mine tailings in arid and semiarid environments: an emerging remediation technology. *Environmental Health Perspectives* **116**: 278–283.

Mendoza-Cózatl DG, Jobe TO, Hauser F, Schroeder JI. 2011. Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. *Current Opinion in Plant Biology* **14**: 554–562.

Merchan F, de Lorenzo L, Ritzzo SG, Niebel A, Manyani H, Frugier F, Sousa C, Crespi M. 2007. Identification of regulatory pathways involved in the reacquisition or root growth after salt stress in *Medicago truncatula. Plant Journal* **51**: 1–17.

Michalak A. 2006. Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. *Polish Journal of Environmental Studies* **15**: 523–530.

Micheli F. 2001. Pectin methylesterases: cell wall enzymes with important roles in plant physiology. *Trends in Plant Science* **6**: 414–419.

Middleton PH, Jakab JL, Penmetsa RV, Starker CG, Doll J, Kaló P, Prabhu R, Marsh JF, Mitra RM, Kereszt A *et al.* 2007. An ERF transcription factor in *Medicago truncatula* that is essential for nod factor signal transduction. *Plant Cell* **19**: 1221–1234.

Mitra RM, Shaw SL, Long SR. 2004. Six non-nodulating plant mutants defective for Nod factor-induced transcriptional changes associated with the legume–rhizobia symbiosis. *Proceedings of the National Academy of Sciences, USA* **101**: 10217–10222.

Moreau S, Verdenaud M, Ott T, Letort S, de Billy F, Niebel A, Gouzy J, de Carvalho-Niebel F, Gamas P. 2011. Transcription reprogramming during root nodule development in *Medicago truncatula. PLoS ONE* **6**: e16463.

Nemeti B, Regonesi ME, Tortora P, Gregus Z. 2010. Polynucleotide phosphorylase and mitochondrial ATP synthase mediate reduction of arsenate to the more toxic arsenite by forming arsenylated analogues of ADP and ATP. *Toxicological Sciences* **117**: 270–281.

Nocito FF, Lancilli C, Crema B, Fourcroy P, Davidian J, Sacchi GA. 2006. Heavy metal stress and sulfate uptake in maize roots. *Plant Physiology* **141**: 1138–1148.

Pajuelo E, Carrasco JA, Romero LC, Chamber MA, Gotor C. 2007. Evaluation of the metal phytoextraction potential of crop legumes. Regulation of the expression of o-acetylserine (thiol) lyase under metal stress. *Plant Biology* **9**: 672–681.

Pajuelo E, Rodríguez-Llorente ID, Dary M, Palomares AJ. 2008. Toxic effects of arsenic on *Sinorhizobium-Medicago sativa* symbiotic interaction. *Environmental Pollution* **154**: 203–211.

Pajuelo E, Rodríguez-LLorente ID, Lafuente A, Caviedes MA. 2011. Legume–Rhizobium symbioses as a tool for bioremediation of heavy metal polluted soils. In: Khan MS, Zaidi A, Goel R, Musarrat J, eds. *Biomanagement of metal contaminated soils. Environmental Pollution, vol. 20*. Heidelberg, Germany: Springer, 95–123.

Passardi F, Cosio C, Penel C, Dunand C. 2005. Peroxidases have more functions than a Swiss army knife. *Plant Cell Reports* **24**: 255–265.

Penmetsa RV, Cook DR. 1997. A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. *Science* **275**: 527–530.

Pickering IJ, Price RC, George MJ, Smith RD, George GN, Salt DE. 2000. Reduction and coordination of arsenic in Indian Mustard. *Plant Physiology* **122**: 1171–1177.

Pietta PG. 2000. Flavonoids as antioxidants. *Journal of Natural Products* **63**: 1035–1042.

Pii Y, Molesini B, Masiero S, Pandolfini T. 2012. The non-specific lipid transfer protein N5 of *Medicago truncatula* is implicated in epidermal stages of rhizobium-host interaction. *BMC Plant Biology* **2012**: 233.

Raab A, Feldmann J, Meharg AA. 2004. The nature of arsenic-phytochelatin complexes in *Holcus lanatus* and *Pteris cretica. Plant Physiology* **134**: 1113–1122.

Raab A, Schat H, Meharg AA, Feldmann J. 2005. Uptake, translocation and transformation of arsenate and arsenite in sunflower (*Helianthus annuus*): formation of arsenic-phytochelatin complexes during exposure to high arsenic concentrations. *New Phytologist* **168**: 551–558.

Rahman MA, Hasegawa H, Rahman MM, Islam MN, Majid MMA, Tasmen A. 2007. Effect of arsenic on photosynthesis, growth and yield of five widely cultivated rice (*Oryza sativa* L.) varieties in Bangladesh. *Chemosphere* **67**: 1072–1079.

Rai A, Tripathi P, Dwivedi S, Dubey S, Shri M, Kumar S, Tripathi PK, Dave R, Kumar A, Singh R *et al.* 2011. Arsenic tolerances in rice (*Oryza sativa*) have a predominant role in transcriptional regulation of a set of genes including sulphur assimilation pathway and antioxidant system. *Chemosphere* **82**: 986–995.

Rao KP, Vani G, Kumar K, Wankhede DP, Misra M, Gupta M, Sinha AK. 2011. Arsenic stress activates MAP kinase in rice roots and leaves. *Archives of Biochemistry and Biophysics* **506**: 73–82.

Rausch T, Wachter A. 2005. Sulfur metabolism: a versatile platform for launching defence operations. *Trends in Plant Science* **10**: 503–509.

Reichman S. 2007. The potential use of the legume–rhizobium symbiosis for the remediation of arsenic contaminated sites. *Soil Biology & Biochemistry* **39**: 2587–2593.

Reid RJ, Gridley K, Kawamata Y, Zhu Y. 2013. Arsenite elicits anomalous sulfur starvation responses in barley. *Plant Physiology* **162**: 401–409.

Requejo R, Tena M. 2005. Proteome analysis of maize roots reveals that oxidative stress is a main contributing factor to plant arsenic toxicity. *Phytochemistry* **66**: 1519–1528.

Salcedo G, Sánchez-Monge R, Barber D, Díaz-Perales A. 2007. Plant non-specific lipid transfer proteins: an interface between plant defence and human allergy. *Biochimica et Biophysica Acta* **1771**: 781–791.

Samac DA, Graham MA. 2007. Recent advances in legume-microbe interactions: recognition, defense response, and symbiosis from a genomic perspective. *Plant Physiology* **144**: 582–587.

Schmöger MEV, Oven M, Grill E. 2000. Detoxification of arsenic by phytochelatins in plants. *Plant Physiology* **122**: 793–802.

Sharma P, Jha AB, Dubey RS, Pessarakli M. 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany* **2012**: 217037.

Sharma SS, Dietz KJ. 2009. The relationship between metal toxicity and cellular redox imbalance. *Trends in Plant Science* **14**: 43–50.

Shin H, Shin HS, Dewbre GR, Harrison MJ. 2004. Phosphate transport in *Arabidopsis*: Pht1-1 and Pht1-4 play a major role in phosphate acquisition from both low- and high-phosphate environments. *Plant Journal* **39**: 629–642.

Singh N, Ma LQ, Srivastava M, Rathinasabapathi B. 2006. Metabolic adaptations to arsenic-induced oxidative stress in *Pteris vittata* L. and *Pteris ensiformis* L. *Plant Science* **170**: 274–282.

Sirpiö S, Allahverdiyeva Y, Suorsa M, Paakkarinen V, Vainonen J, Battchikova N, Aro EM. 2007. TLP18.3, a novel thylakoid lumen protein regulating photosystem II repair cycle. *Biochemical Journal* **406**: 415–425.

Smedley PL, Kinniburgh DG. 2002. A review of the source, behaviour and distribution of arsenic in natural waters. *Applied Geochemistry* **17**: 517–568.

Smith SE, Christophersen HM, Pope S, Smith FA. 2010. Arsenic uptake and toxicity in plants: integrating mycorrhizal influences. *Plant and Soil* **327**: 1–21.

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

Song WY, Park J, Mendoza-Cózatl DG, Suter-Grotemeyer M, Shim D, Hörtensteiner S, Geisler M, Weder B, Rea PA, Rentsch D *et al.* 2010. Arsenic tolerance in *Arabidopsis* is mediated by two ABCC-type phytochelatin transporters. *Proceedings of the National Academy of Sciences, USA* **107**: 21187–21192.

Soto MJ, Domínguez-Ferreras A, Pérez-Mendoza D, Sanjuan J, Olivares J. 2009. Mutualism versus pathogenesis: the give-and-take in plant–bacteria interactions. *Cellular Microbiology* **11**: 381–388.

Srivastava S, Mishra S, Tripathi RD, Dwivedi S, Trivedi PK, Tandon PK. 2007. Phytochelatins and antioxidant systems respond differentially during arsenite and arsenate stress in *Hydrilla verticillata* (L.f.) Royle. *Environmental Science and Technology* **41**: 2930–2936.

Srivastava S, Srivastava AK, Suprasanna P, D'Souza SF. 2009. Comparative biochemical and transcriptional profiling of two contrasting varieties of *Brassica juncea* L. in response to arsenic exposure reveals mechanisms of stress perception and tolerance. *Journal of Experimental Botany* **60**: 3419–3431.

Srivastava S, Srivastava AK, Suprasanna P, D'Souza SF. 2013. Identification and profiling of arsenic stress-induced microRNAs in *Brassica juncea*. *Journal of Experimental Botany* **64**: 303–315.

Stracke R, Werber M, Weisshaar B. 2001. The *R2R3-MYB* gene family in *Arabidopsis thaliana*. *Current Opinion in Plant Biology* **4**: 447–456.

Szabados L, Savouré A. 2010. Proline: a multifunctional amino acid. *Trends in Plant Science* **15**: 89–97.

Talano MA, Cejas RB, Gonzalez PS, Agostini E. 2012. Arsenic effect on the model crop symbiosis *Bradyrhizobium*-soybean. *Plant Physiology and Biochemistry* **63**: 8–14.

Terpolilli JJ, O'Hara GW, Tiwari RP, Dilworth MJ, Howieson JG. 2008. The model legume *Medicago truncatula* A17 is poorly matched for N_2 fixation with the sequenced microsymbiont *Sinorhizobium meliloti* 1021. *New Phytologist* **179**: 62–66.

Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, Selbig J, Muller LA, Rhee SY, Stitt M. 2004. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *Plant Journal* **37**: 914–939.

Toledo-Ortiz G, Huq E, Quail PH. 2003. The *Arabidopsis* basic/helix-loop-helix transcription factor family. *Plant Cell* **15**: 1749–1770.

Tripathi P, Mishra A, Dwivedi S, Chakrabarty D, Trivedi PK, Singh RP, Tripathi RD. 2012a. Differential response of oxidative stress and thiol metabolism in contrasting rice genotypes for arsenic tolerance. *Ecotoxicology and Environmental Safety* **79**: 189–198.

Tripathi RD, Tripathi P, Dwivedi S, Dubey S, Chatterjee S, Chakrabarty D, Trivedi PK. 2012b. Arsenomics: omics of arsenic metabolism in plants. *Frontiers in Physiology* **3**: 275.

Tuan LQ, Huong TTT, Hong PTA, Kawakami T, Shimanouchi T, Umakoshi H, Kuboi R. 2008. Arsenic (V) induces a fluidization of algal cell and liposome membranes. *Toxicology in Vitro* **22**: 1632–1638.

Ullrich-Eberius CI, Sanz A, Novacky AJ. 1989. Evaluation of arsenate- and vanadate-associated changes of electrical membrane potential and phosphate transport in *Lemna gibba*-G1. *Journal of Experimental Botany* **40**: 119–128.

Usadel B, Nagel A, Thimm O, Redestig H, Blaesing OE, Palacios-Rojas N, Selbig J, Hannemann J, Pigues MC, Steinhauser D *et al.* 2005. Extension of the visualization tool MapMan to allow statistical analysis of arrays, display of corresponding genes, and comparison with known responses. *Plant Physiology* **138**: 1195–1204.

Van de Mortel JE, Schat H, Moerland PD, Ver Loren van Themaat E, van der Ent S, Blankestijn H, Ghandilyan A, Tsiatsiani S, Aarts MG. 2008. Expression differences for genes involved in lignin, glutathione and sulfate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd-hyperacc-
umulator *Thlaspi caerulescens*. *Plant, Cell & Environment* **31**: 301–324.

Van de Velde W, Zehirov G, Szatmari A, Debreczeny M, Ishihara H, Kevei Z, Farkas A, Mikulass K, Nagy A, Tiricz H *et al.* 2010. Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science* **327**: 1122–1126.

VandenBosch K, Endre G, Hur J, Moore J, Beremand P, Ellis L, Town CD, Bowman CL, Craven MB, Hansen TS *et al.* 2000. ESTs from roots of *Medicago truncatula* 24 hours after inoculation with *Sinorhizobium meliloti*. EMBL accession no. BF004720.

Vázquez S, Agha R, Granado A, Sarro MJ, Esteban E, Peñalosa JM, Carpena RO. 2006. Use of white lupin plant for phytostabilization of Cd and As polluted acid soil. *Water, Air, and Soil pollution* **177**: 349–365.

Vázquez S, Esteban E, Carpena RO. 2008. Evolution of arsenate toxicity in nodulated white lupine in a long-term culture. *Journal of Agriculture and Food Chemistry* **56**: 8580–8587.

Verdnik D. 2004. *Guide to microarray analysis*. Sunnyvale, CA, USA: MDS Analytical Technologies. URL: https://www.natur.cuni.cz/biologie/servisni-laboratore/genomicka-a-proteomicka-laborator/soubory/guide-to-microarray-analysis

Vogt T. 2010. Phenylpropanoid biosynthesis. *Molecular Plant* **3**: 2–20.

Wang Q, He M, Wang Y. 2011. Influence of combined pollution of antimony and arsenic on culturable soil microbial populations and enzyme activities. *Ecotoxicology* **20**: 9–19.

Wangeline AL, Burkhead JL, Hale KL, Lindblom SD, Terry N, Pilon M, Pilon-Smits EAH. 2004. Overexpression of ATP sulfurylase in Indian mustard: effects on tolerance and accumulation of twelve metals. *Journal of Environmental Quality* **33**: 54–60.

Wani PA, Khan MS, Zaidi A. 2008. Chromium-reducing and plant growth-promoting *Mesorhizobium* improves chickpea growth in chromium-amended soil. *Biotechnology Letters* **30**: 159–163.

Winkel-Shirley B. 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell Biology, and biotechnology. *Plant Physiology* **126**: 485–493.

Xiao H, Yin L, Xu X, Li T, Han Z. 2008. The iron-regulated transporter, MbNRAMP1, isolated from *Malus baccata* is involved in Fe, Mn and Cd trafficking. *Annals of Botany* **102**: 881–889.

Xiao W, Jang JC. 2000. F-box protein in *Arabidopsis*. *Trends in Plant Science* **5**: 454–457.

Xu XY, McGrath SP, Meharg AA, Zhao FJ. 2008. Growing rice aerobically markedly decreases arsenic accumulation. *Environmental Science and Technology* **42**: 5574–5579.

Yang HC, Cheng J, Finan TM, Rosen BP, Bhattacharjee H. 2005. Novel pathway for arsenic detoxification in the legume symbiont *Sinorhizobium meliloti*. *Journal of Bacteriology* **187**: 6991–6997.

Yano K, Shibata S, Chen WL, Sato S, Kaneko T, Jurkiewicz A, Sandal N, Banba M, Imaizumi-Anraku H, Kojima T *et al.* 2009. CERBERUS, a novel U-box protein containing WD-40 repeats, is required for formation of the infection thread and nodule development in the legume–*Rhizobium* symbiosis. *Plant Journal* **60**: 168–180.

Zaidi A, Wani PA, Khan MS. 2012. *Toxicity of heavy metals to legumes and bioremediation*. Wein, Germany: Springer.

Zélicourt A, Diet A, Marion J, Laffont C, Ariel F, Moison M, Zahaf O, Crespi M, Gruber V, Frugier F. 2012. Dual involvement of a *Medicago truncatula* NAC transcription factor in root abiotic stress response and symbiotic nodule senescence. *Plant Journal* **70**: 220–230.

Zhao FJ, Ma JF, Meharg AA, McGrath SP. 2009. Arsenic uptake and metabolism in plants. *New Phytologist* **181**: 777–794.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Split-root system developed for analysing the effect of As (III) on nodulation in one side of the roots of *Medicago truncatula* and root elongation in the other side.

Table S1 List of primers used for qRT-PCR and for generation of *Medicago truncatula* composite plants

Table S2 List of *Medicago truncatula* genes that were up- or down-regulated more than 5-fold in hybridization 1

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

Table S3 List of *Medicago truncatula* genes that were up- or down-regulated more than 4-fold in hybridization 2

Table S4 List of *Medicago truncatula* genes that were up- or down-regulated more than 3-fold in hybridization 3

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.

About *New Phytologist*

- *New Phytologist* is an electronic (online-only) journal owned by the New Phytologist Trust, a **not-for-profit organization** dedicated to the promotion of plant science, facilitating projects from symposia to free access for our Tansley reviews.
- Regular papers, Letters, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication 'as ready' via *Early View* – our average time to decision is <25 days. There are **no page or colour charges** and a PDF version will be provided for each article.
- The journal is available online at Wiley Online Library. Visit **www.newphytologist.com** to search the articles and register for table of contents email alerts.
- If you have any questions, do get in touch with Central Office (np-centraloffice@lancaster.ac.uk) or, if it is more convenient, our USA Office (np-usaoffice@lancaster.ac.uk)
- For submission instructions, subscription and all the latest information visit **www.newphytologist.com**

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust