Journal of
Experimental
Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Thiol synthetases of legumes: immunogold localization and differential gene regulation by phytohormones

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Abstract

In plants and other organisms, glutathione (GSH) biosynthesis is catalysed sequentially by γ-glutamylcysteine synthetase (γECS) and glutathione synthetase (GSHS). In legumes, homoglutathione (hGSH) can replace GSH and is synthesized by γECS and a specific homoglutathione synthetase (hGSHS). The subcellular localization of the enzymes was examined by electron microscopy in several legumes and gene expression was analysed in *Lotus japonicus* plants treated for 1–48 h with 50 μM of hormones. Immunogold localization studies revealed that γECS is confined to chloroplasts and plastids, whereas hGSHS is also in the cytosol. Addition of hormones caused differential expression of thiol synthetases in roots. After 24–48 h, abscisic and salicylic acids downregulated *GSHS* whereas jasmonic acid upregulated it. Cytokinins and polyamines activated *GSHS* but not *γECS* or *hGSHS*. Jasmonic acid elicited a coordinated response of the three genes and auxin induced both hGSHS expression and activity. Results show that the thiol biosynthetic pathway is compartmentalized in legumes. Moreover, the similar response profiles of the GSH and hGSH contents in roots of non-nodulated and nodulated plants to the various hormonal treatments indicate that thiol homeostasis is independent of the nitrogen source of the plants. The differential regulation of the three mRNA levels, hGSHS activity, and thiol contents by hormones indicates a fine control of thiol biosynthesis at multiple levels and strongly suggests that GSH and hGSH play distinct roles in plant development and stress responses.

Key words: γ-Glutamylcysteine synthetase, (homo)glutathione synthetase, immunogold localization, legumes, phytohormones, plant stress.

Introduction

The thiol tripeptide glutathione (GSH; γGlu–Cys–Gly) is a major water-soluble antioxidant and redox buffer in plants, animals, and microorganisms (Meister, 1994; Wild and Mulcahy, 2000; Foyer and Noctor, 2011). In plants, GSH also performs critical functions in cell cycle regulation, plant development, sulphur transport and storage, stress response, and heavy metal detoxification (Maughan and Foyer, 2006). In legumes, the structurally related tripeptide homoglutathione (hGSH; γGlu–Cys–βAla) may partially or completely replace GSH with presumably the same functions (Frendo *et al.*, 2001; Matamoros *et al.*, 2003).

The synthesis of GSH is accomplished in two sequential ATP-dependent reactions catalysed by γ-glutamylcysteine synthetase (γECS) and glutathione synthetase (GSHS), whereas the synthesis of hGSH shares the same first enzyme and then requires a specific homoglutathione synthetase (hGSHS). The biochemical properties of the three thiol synthetases have been determined (Macnicol, 1987; Hell and Bergmann, 1990; Iturbe-Ormaetxe *et al.*, 2002; Jez and

Cahoon, 2004). However, there are still uncertainties about their subcellular localizations. Early reports using purified organelles from leaves of spinach (*Spinacia oleracea*), pea (*Pisum sativum*), and runner bean (*Phaseolus coccineus*) concluded that the three enzymes are located in the chloroplasts and cytosol (Klapheck *et al.*, 1988; Hell and Bergmann, 1990), but subsequent studies with nodules and leaves of common bean (*Phaseolus vulgaris*) and cowpea (*Vigna unguiculata*) reported that γECS is in the chloroplasts and plastids and that at least some GSHS and hGSHS isoforms are present in the cytosol of nodule host cells (Moran *et al.*, 2000). Recently, cellular and molecular analyses have also indicated that, in *Arabidopsis*, γECS is localized exclusively in the plastids, whereas GSHS occurs as a mixture of plastidic and cytosolic isoforms that are encoded by two transcript populations of the same gene (Wachter *et al.*, 2005). The more sensitive and precise technique of immunogold electron microscopy has not been used so far to study the subcellular localization of thiol synthetases and, in particular, of hGSHS in legume tissues.

Likewise, information on the regulation of the genes involved in thiol biosynthesis is scarce and in some cases contradictory. Expression of *γECS* and *GSHS* remains invariant in *Arabidopsis* suspension cell cultures exposed to cadmium or xenobiotics that elicit a rapid accumulation of GSH (May *et al.*, 1998). In contrast, treatment of *Arabidopsis* with metals known to mobilize GSH for phytochelatin synthesis increased coordinately the transcription of *γECS* and *GSHS* (Xiang and Oliver, 1998). A strong increase in *γECS* expression was also observed in leaves and roots of Indian mustard (*Brassica juncea*) exposed to cadmium (Schäfer *et al.*, 1998; Wachter *et al.*, 2005). Even less is known about the control of *hGSHS* expression. Only two reports have examined to date the effects of environmental cues or signal molecules on *hGSHS* expression. Thus, treatment of *Medicago truncatula* plants with compounds that release nitric oxide (NO), a key signalling molecule in plants (Neill *et al.*, 2003), induced expression of *γECS* and *GSHS*, but not of *hGSHS*, in roots (Innocenti *et al.*, 2007). Similarly, common bean plants treated with H_2O_2 showed upregulation of *γECS* and *hGSHS* in nodules, whereas treatments with cadmium, sodium chloride, or jasmonic acid (JA) had no effect (Loscos *et al.*, 2008).

A better understanding of the regulation of GSH and hGSH biosynthesis in legumes during the stress response requires a precise determination of the subcellular localization of the enzymes and a quantitative expression analysis of the genes involved. In the present work, two objectives were pursued. First, polyclonal antibodies against γECS and hGSHS were produced to immunolocalize both proteins in legumes, taking advantage of the superior resolution of electron microscopy over subcellular fractionation or light microscopy localization techniques. Second, the expression pattern of the three thiol synthetase genes was determined in the model legume *Lotus japonicus* supplied with several hormones and related compounds that are involved in stress signalling (Fujita *et al.*, 2006; Balbi and

Devoto, 2008). This part of the study was focused on roots as they responded more rapidly to hormones than the leaves and it avoided the complication of different rates of hormone transport to the shoot. Nodulated plants were included to determine whether the nodulation status could alter the response of thiol synthesis to the hormonal treatments. These experiments were of interest because ethylene, ABA, JA, and SA inhibit nodulation, possibly as a mechanism to control nodule number (Stacey *et al.*, 2006; Sun *et al.*, 2006; Ding *et al.*, 2008; Tominaga *et al.*, 2009), whereas CK activates nodule formation (González-Rizzo *et al.*, 2006; Tirichine *et al.*, 2007).

Materials and methods

Plant growth and treatments

Nodulated plants of alfalfa (*Medicago sativa* L. cv. Aragón × *Sinorhizobium meliloti* 102F78) and common bean (*P. vulgaris* L. cv. Contender × *Rhizobium leguminosarum* bv. *phaseoli* 3622) were grown for 50–55 or 28–30 d, respectively, in pots containing vermiculite under controlled environment conditions (Naya *et al.*, 2007; Loscos *et al.*, 2008). Non-nodulated and nodulated plants of *L. japonicus* (Regel) Larsen ecotype MG-20 were grown for 21 and 45 d, respectively, in aerated hydroponic cultures under controlled environment conditions. The two sets of plants were harvested at different ages to compensate for the slower growth of nodulated plants; hence, they had similar weights and physiological ages to non-nodulated plants. Nodules of *L. japonicus* were produced by inoculation of seedling roots with *Mesorhizobium loti* R7A. The hydroponic medium was 4 l of 1/4 strength B&D nutrient solution (Broughton and Dilworth, 1971), containing 0 or 1.25 mM NH_4NO_3 for nodulated or non-nodulated plants, respectively. Root and stem nodules of *Sesbania rostrata* were produced by inoculation with *Azorhizobium caulinodans* ORS 571, and plants were grown in pots with vermiculite in a glasshouse for 30 d (James *et al.*, 1996). All leguminous plants were at the vegetative stage when leaves, roots, and nodules were harvested. Plant material to be used for expression analysis of *γECS*, *GSHS*, and *hGSHS* was flash frozen in liquid nitrogen and stored at –80 °C, whereas material to be used for immunolocalization studies was immediately high-pressure frozen (see below).

To investigate the effects of hormones on expression of thiol synthetase genes, *L. japonicus* plants were treated for up to 48 h with 50 μM of abscisic acid (ABA), gibberellic acid (GA), salicylic acid (SA), JA, indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC; the immediate ethylene precursor), cytokinins (CK; an equimolar mixture of kinetin and 6-benzylaminopurine), or polyamines (PA; an equimolar mixture of spermine, spermidine, and putrescine). Stock solutions of compounds (Sigma-Aldrich) were prepared as follows: 500 mM kinetin and 500 mM 6-benzylaminopurine (each in 200 μl of 1 M NaOH); 500 mM IAA (in 400 μl of 1 M NaOH); 100 mM ABA, ACC, PA, or SA (in 2 ml of ethanol); and 100 mM JA or GA (in 2 ml of dimethylsulphoxide). These volumes were then added to 4 l of the hydroponic solution, which was maintained at pH 6.6 for all treatments. Control plants that had grown simultaneously in hydroponics, and that had been treated with identical concentrations of NaOH, ethanol, or dimethylsulphoxide at the same time points, were used to correct gene expression values of the hormone treatments. Nutrient solution in hydroponics was maintained fully aerated during all the experiments by bubbling air at a flow rate of 160 l h^{-1} with a Rena Air 200 aquarium pump (Chalfont, Pennsylvania, USA).

To assesss the effects of NO on gene expression, *L. japonicus* plants were grown for 15 d in 1.5% agar plates (8–10 seedlings per

10 × 10-cm square plate) on modified Fahraeus medium without nitrogen (Boisson-Dernier *et al.*, 2001). Plates were placed vertically under the same controlled environment conditions mentioned above, except that they were placed in the dark during the 24-h treatment. The plates contained a filter paper between the agar and the plants to maintain humidity and to avoid roots entering the agar. The NO-releasing compound *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP; Sigma-Aldrich) was added to the nutrient solution at a concentration of 500 μM, and plants were harvested after 3 and 24 h. The nutrient solution covered only about one-third of the rooting system to prevent anoxia.

Production and purification of recombinant enzymes

The open reading frame of common bean γECS without the signal peptide was amplified by PCR using specific primers (forward, 5'-CCATGGCGAGCCCGCCCACTG-3'; reverse, 5'-GCGGCCGC-TAAGACACCCTTAATAAAG-3'). The product was cloned into the pCRII vector (Invitrogen) and the amplified fragment was digested with *Nco*I and *Pst*I and cloned in a modified expression vector (pMAL-c2*). This plasmid was derived from pMAL-c2 (New England Biolabs, Beverly, USA) by including, within the *Xmn*I multiple cloning site, a 6 × His coding sequence, a thrombin cleavage site, and a *Nco*I site. The construct in *Escherichia coli* DH5α cells was sequenced to verify the absence of errors in the open reading frame and was then transferred to BL21(DE3) cells to express the recombinant protein. The fusion protein contained the maltose-binding protein at the N-terminus, followed by the 6 × His tag and the mature γECS protein.

To purify enough recombinant protein for antibody production, cultures (500 ml) were inoculated with 1 ml of a preculture (LB medium with ampicillin) of the recombinant clone that had been grown overnight, and cells were grown at 37 °C until the absorbance at 600 nm reached 0.7–0.8. Expression was then induced with 250 μM isopropyl β-D-1-thiogalactopyranoside for 2 h at 37 °C. Cells were harvested by centrifugation and washed with 20 mM sodium phosphate (pH 7.4) and 0.5 M NaCl, and the pellet was resuspended in 5 ml of wash medium, frozen in liquid nitrogen, and stored at –80 °C. The cell suspension was thawed at 37 °C and sonicated (6 × 30 s). Lysed cells were centrifuged in the cold and the pellet was resuspended in 5 ml of 20 mM sodium phosphate (pH 7.4), 0.5 M NaCl, 25 mM imidazole, and 6 M guanidine. After centrifugation, the supernatant was saved and the pellet was resuspended in 5 ml of the same medium. This suspension was sonicated for another 30 s and centrifuged, and the supernatants were pooled.

The recombinant protein was purified in a single step from the pooled supernatants by using a HiTrap Chelating HP (5 ml) column, previously loaded with 100 mM NiSO$_4$ and then washed with two volumes of water, essentially as recommended by the manufacturer (GE Healthcare Bio-Sciences, Uppsala, Sweden). The lyophilized protein (~6–7 mg) was used to immunize two rabbits and to prepare an affinity column for purification of the monospecific antibody from the antiserum following conventional protocols (BioGenes, Berlin, Germany). Briefly, the protein was coupled to CNBr-activated Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences), the antiserum was loaded on the column, and the monospecific IgG was eluted with 200 mM Gly-HCl buffer containing 250 mM NaCl (pH 2.2). The eluate was immediately adjusted to pH 7.5 with 2 M TRIS-HCl.

A similar procedure was followed to prepare recombinant hGSHS protein using sequence information of pea hGSHS (Iturbe-Ormaetxe *et al.*, 2002) and to purify the corresponding polyclonal antibody.

Immunoblot analyses and immunolocalization of thiol synthetases

Immunoblots to monitor purification of recombinant proteins were performed by using a monoclonal antibody (clone His-1) against the His tag as the primary antibody (dilution 1:3000) and goat anti-mouse IgG conjugated to alkaline phosphatase as the secondary antibody (dilution 1:30000), as described by the supplier (Sigma-Aldrich). Immunoreactive proteins were detected with alkaline phosphatase substrate containing 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Sigma-Aldrich).

Immunoblots of plant extracts were performed according to published procedures (Rubio *et al.*, 2009). The secondary antibody was a goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma-Aldrich). The primary antibody was used at a dilution of 1:1000 (γECS) or 1:250 (hGSHS) and the secondary antibody at a dilution of 1:20000 (γECS and hGSHS). Immunoreactive proteins were detected by chemiluminescence using the SuperSignal West Pico or SuperSignal West Femto kits (Thermo Scientific, Rockford, IL, USA).

For immunogold localization, plant material was high-pressure frozen using an EM-PACT (Leica) instrument, and then freeze-substituted and embedded in low-temperature resin (Lowicryl HM23, Polysciences, Warrington, PA, USA) using an EM-AFS (Leica). Details of these procedures were given elsewhere (Rubio *et al.*, 2009).

Expression analyses of thiol synthetases

Total RNA was extracted from roots and leaves with the RNAqueous kit (Ambion, Austin, TX, USA), and mRNA levels were determined by quantitative reverse-transcription PCR analysis using gene-specific primers as described and *ubiquitin* as the reference gene (Matamoros *et al.*, 2003). The PCR amplification products were confirmed by melting curve analysis and the primer efficiencies, calculated by serial dilutions, were >90%. The number of amplification cycles with respect to *ubiquitin* (ΔC_t) were ~7–10 for γECS and hGSHS and ~12–15 for GSHS.

Thiol synthetase activities and thiol contents

Thiol synthetase activities were determined by quantifying the GSH and hGSH produced by GSHS and hGSHS, respectively (Hell and Bergmann, 1988; Matamoros *et al.*, 1999). The enzymes were extracted at 4 °C from 100 mg of roots with 500 μl of a medium consisting of 50 mM TRIS-HCl (pH 8.0), 0.2 mM EDTA, 10 mM MgCl$_2$, and 10% glycerol. The extracts were cleared by centrifugation and depleted of thiols and other endogenous low molecular mass compounds using Vivaspin (10 kDa cut off) ultrafiltration devices (Sartorius, Goettingen, Germany). The reaction mixtures (final volume of 200 μl) contained 100 mM TRIS-HCl (pH 8.5), 50 mM KCl, 20 mM MgCl$_2$, 5 mM ATP, 5 mM phospho*enol*pyruvate, 5 units of pyruvate kinase, 5 mM dithioerythritol, 0.5 mM γ-glutamylcysteine, 5 mM Gly (GSHS) or β-Ala (hGSHS), and 100 μl of extract to initiate the reaction. This was terminated after 0 or 60 min at 30 °C by transferring 80-μl aliquots to derivatizing solution, which comprised 300 μl of 200 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(3-propanesulphonic acid) and 5 mM diethylenetriaminepenta-acetic acid (EPPS/DTPA buffer, pH 8.0), and 120 μl of 7 mM monobromobimane (MBB; Calbiochem). The samples were further incubated for 15 min in the dark and derivatization was stopped by adding 97 μl of 40% acetic acid. Samples were kept at –80 °C until analysis, which was performed by HPLC with fluorescence detection as previously described (Matamoros *et al.*, 1999).

Thiol tripeptides (GSH and hGSH) were extracted from 100 mg of roots with 200 μl of 200 mM methanesulphonic acid containing 0.5 mM DTPA. The extracts were cleared by centrifugation and 50 μl of supernatant was mixed with 23 μl of 4 mM dithioerythritol, 100 μl of EPPS/DTPA buffer (pH 8.0), and 2 μl of 5 M NaOH. The mix was incubated for 1 h at room temperature and 50 μl of 7 mM MBB was added and left for 15 min in the dark. Derivatization was stopped by adding 90 μl of 20% acetic acid. The samples were centrifuged and the thiol derivatives were quantified by HPLC with fluorescence detection (Matamoros *et al.*, 1999). The low concentrations of GSH were accurately determined by HPLC coupled to mass spectrometry (MS). Samples were analysed by liquid chromatography-tandem MS (LC-MS/MS) using a linear LTQ ion trap equipped with a micro-electrospray ionization source (Thermo-Fisher, San Jose, CA, USA). A 20 μl-aliquot was diluted to 40 μl

with 1% formic acid prior to instrumental analysis and loaded on a chromatographic system consisting of a C_{18} preconcentration cartridge (Agilent Technologies, Barcelona) connected to a 10 cm long × 150 μm i.d. C_{18} column (Vydac, IL, USA). The separation was done at 1 μl min^{-1} in a 30 min acetonitrile 0–40% gradient (solvent A: 0.1% formic acid; solvent B: acetonitrile with 0.1% formic acid). The HPLC system comprised an Agilent 1200 capillary pump, binary pump, thermostated microinjector, and microswitch valve. The LTQ instrument was operated in the positive ion mode with a spray voltage of 2 kV. The spectrometric analysis was performed in a targeted mode, acquiring a full MS/MS scan of the precursor ions of GSH (m/z=498.2) and hGSH (m/z=512.2). The quantification was performed using extracted ion chromatograms of the optimum MS/MS transitions in terms of sensitivity (GSH, 498.2→435.2; hGSH, 512.2→449.2).

Results

Subcellular localization of thiol synthetases in legumes

To perform immunolocalization studies of thiol synthetases in leaves, roots, and nodules of some crop and model legumes, it was necessary to purify the proteins and generate antibodies. Preliminary attempts to purify the enzymes directly from legume tissues were unsuccessful as they were found at a low concentration and γECS was particularly labile during extraction. This could explain the lack of any previous immunolocalization of thiol synthetases. Thus, γECS and hGSHS were expressed in *E. coli* as fusion proteins to enhance their expression and/or solubility. The recombinant proteins had a His tag and were purified with metal-chelating columns. The presence of several protein bands in the preparation of purified γECS (Fig. 1A) can be detected when using the pMAL expression system and is not due to contaminants but to the formation of truncated proteins by partial proteolysis of the fusion protein (Riggs, 2000). This can also be inferred from the fact that these proteins contain the His tags (lane PP, Fig. 1A). In addition, a protein band of ~50 kDa was observed in the induced and soluble fractions (Fig. 1A, lanes I and S), which was attributed to the maltose-binding protein-tagged protein based on its expected molecular mass and high solubility and stability (Riggs, 2000). This protein product may have originated by proteolysis of the whole fusion protein but its identity was not verified. The fusion
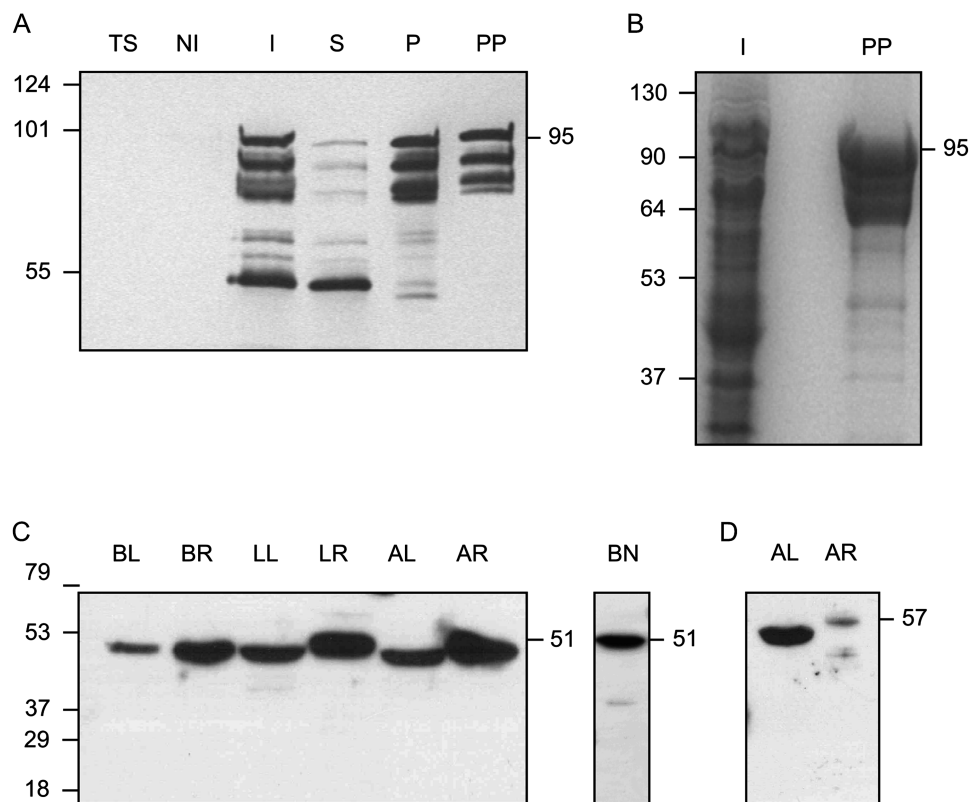
Fig. 1. Expression and purification of γECS and immunoblots of γ-glutamylcysteine synthetase (γECS) and homoglutathione synthetase (hGSHS) in legumes. (A) Purification of the fusion protein between the γECS from bean and the maltose-binding protein from *Escherichia coli*. Immunoblot using a monoclonal antibody against the His tag: TS, untransformed *E. coli* BL21 cells (10 μg protein); NI, non-induced transformed cells (10 μg protein); I, induced transformed cells (10 μg protein); S, supernatant of lysed induced cells (10 μg protein); P, pellet of lysed induced cells (2.5 μg protein); PP, purified recombinant enzyme (0.5 μg protein). (B) SDS gel stained with Coomassie. I, induced transformed cells (50 μg protein); PP, purified recombinant enzyme (10 μg protein). The expected molecular mass of the fusion protein is ~95 kDa. (C, D) Immunoblots of γECS in several organs of representative legumes (C) and hGSHS in alfalfa (D) (20 μg protein). BL, bean leaves; BR, bean roots; BN, bean nodules; LL, *Lotus japonicus* leaves; LR, *Lotus japonicus* roots; AL, alfalfa leaves; AR, alfalfa roots. Detection was by chemiluminescence with the SuperSignal West Pico (A–C) or Femto (D) kits. Molecular masses (kDa) of the protein markers are shown on the left and apparent molecular masses (kDa) of the proteins are given on the right.

protein containing γECS accounted for >95% of the total protein, judging from densitometric analysis of the Coomassie-stained gel (Fig. 1B, lane PP). Recombinant γECS and hGSHS were used to produce antisera, and the polyclonal monospecific antibodies were affinity purified. The γECS antibody recognized a single protein band (51 kDa) in extracts of leaves and roots of all legumes examined; the same immunoreactive protein band could be observed in nodules (Fig. 1C).

However, the hGSHS antibody recognized a single protein band at the expected mass (~57 kDa) in extracts of alfalfa leaves and roots (Fig. 1D) but the corresponding immunoreactive protein was not seen in extracts of *L. japonicus* or common bean (data not shown). Therefore, immunogold localization studies of γECS were carried out with several legumes but those of hGSHS were limited to alfalfa. The hGSHS protein band in alfalfa leaves was clearly more abundant than in roots and the protein in roots showed a slightly higher apparent molecular mass (Fig. 1D). Because the amino acid sequences of GSHS and hGSHS in both *M. truncatula* and *L. japonicus* share 77% identity and a similar value is expected for the two proteins of alfalfa, the possibility cannot be ruled out that the hGSHS antibody also recognizes GSHS. However, hGSHS activity is ~10–13-fold higher than GSHS activity in alfalfa leaves (4.86 ± 0.27 *versus* 0.50 ± 0.05 nmol min^{-1} (g fresh weight)$^{-1}$) and roots (6.87 ± 1.33 *versus* 0.53 ± 0.10 nmol min^{-1} (g fresh weight)$^{-1}$). Therefore, it is concluded that the antibody recognizes hGSHS and that GSHS is present at negligible amounts in alfalfa leaves and roots.

The novel immunolocalization of γECS and hGSHS in legumes entailed sample processing by high-pressure freezing, freeze substitution, and embedding at low temperature. This method optimizes the preservation of protein epitopes in leaves and nodules, thus allowing for a more precise immunolocalization (Rubio *et al.*, 2009). For γECS immunolocalization, two representative crop legumes (common bean and alfalfa) and two model legume species (*S. rostrata* and *L. japonicus*) were selected. The tropical legume *S. rostrata* was also included in this study because it is a model for stem nodulation and the immunolocalization of γECS in the photosynthetic stem nodules was of interest in relation to O$_2$ regulation (James *et al.*, 1996). All three typical plant organs (roots, nodules, and leaves) were examined in detail for most of these species with identical results. Therefore, only a summary of results is presented in Fig. 2. The γECS protein was localized in the amyloplasts of common bean roots (Fig. 2A) and nodules (Fig. 2B). Immunolabelling was also observed in the amyloplasts of *S. rostrata* root nodules (Fig. 2C) and in the chloroplast thylakoid membranes of stem nodules (Fig. 2D). In alfalfa leaves, γECS was localized to the chloroplasts and much of the labelling was on the starch grains as well as on the thylakoid membranes (Fig. 2E). As a negative control, preimmune serum was used instead of the antibody and in this case no labelling was observed (Fig. 2F). The hGSHS protein was mainly localized on starch grains within chloroplasts of alfalfa leaves (Fig. 3A, B) and plastids of alfalfa

roots (Fig. 3C), although there was some sparse labelling within the cytoplasm (Fig. 3A–C). No labelling was detected when the antibody was substituted for preimmune serum (Fig. 3D).

Transcriptional regulation of thiol synthetases in response to hormones and nitric oxide

A first type of experiment, aimed at investigating the short-term transcriptional regulation of the thiol biosynthetic pathway, was conducted by exposing *L. japonicus* plants to hormones and stress-related compounds. This legume species was chosen because its thiol synthetase genes have been characterized and their expression levels determined in various plant tissues (Matamoros *et al.*, 2003). Hormones were provided to plants in the hydroponic medium at a physiologically relevant concentration and the mRNA levels of the three thiol synthetases were quantified in roots, the initial target of hormonal action in the time frame of a few hours. In order to keep these time-course experiments within manageable limits, three hormones (ABA, SA, and JA) were applied to non-nodulated plants (Fig. 4A) and another three hormones (IAA, CK, and PA) to nodulated plants (Fig. 4B). As will be described later, this study was nevertheless complemented with other experiments in which each of the six hormones was provided for 48 h to both non-nodulated and nodulated plants. Initial studies also included GA and ACC, but these compounds were found not to have any meaningful effect on gene expression in roots of non-nodulated plants (data not shown).

The exposure of roots to SA did not affect *γECS* mRNA levels, slightly upregulated *hGSHS* after 1–3 h, and strongly downregulated *GSHS* and *hGSHS* after 24 h (Fig. 4A). In sharp contrast, JA triggered a coordinated response of the *γECS*, *GSHS*, and *hGSHS* mRNA levels. Thus, JA caused upregulation of the three genes after 1 h of treatment, and this induction was followed by a transient downregulation after 3 or 6 h and by the subsequent recovery of mRNA levels to at least control values after 24 h (Fig. 4A). The hormones ABA and PA are major components of the signalling network for abiotic stress (Bouchereau *et al.*, 1999; Fujita *et al.*, 2006). However, they affected differently the expression of thiol synthetase genes in the roots (Fig. 4). The application of ABA resulted in upregulation of *γECS* after 6–24 h and in downregulation of *GSHS* and *hGSHS* after 24 h. By contrast, exogenous supply of PA to the rooting medium had very minor or no effects on *γECS* and *hGSHS* mRNA levels, but strongly activated *GSHS* after 24 h. In plants, auxins and CK are required, among other functions, for the development of root and shoot meristems (Dello Ioio *et al.*, 2008). In the short-term, the application of IAA and CK increased the *GSHS* mRNA level by ~10-fold and 3-fold, respectively, and had virtually no effects on the other two genes (Fig. 4B).

Because several hormones, including ABA and PA, are known to induce NO synthesis (Neill *et al.*, 2003; Tun *et al.*, 2006), the effects of an NO-releasing compound, SNAP, on the mRNA levels of thiol synthetases were examined. The
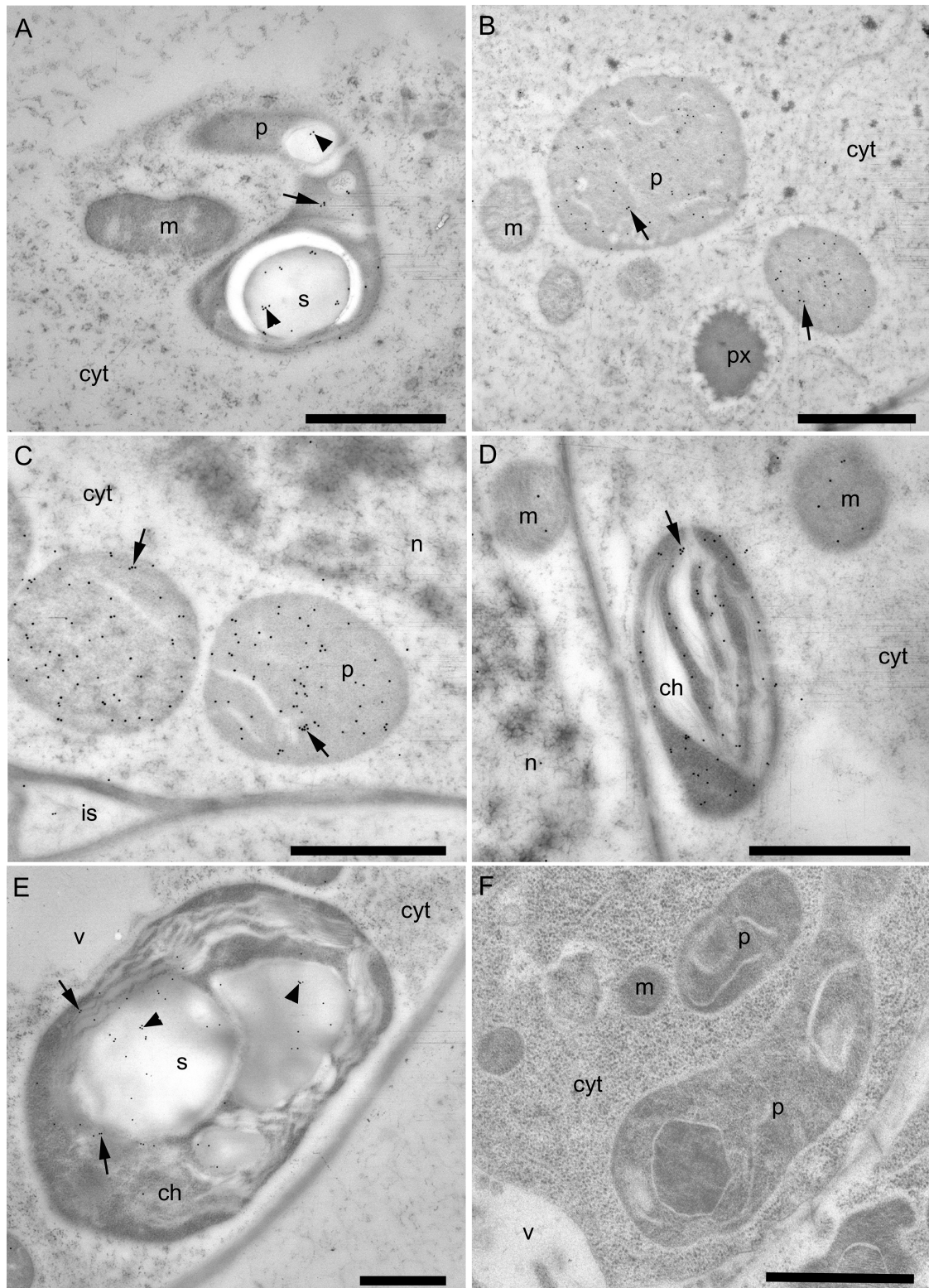
Fig. 2. Immunogold localization of γ-glutamylcysteine synthetase (γECS) in roots, nodules, and leaves of several legumes.
(A) Immunogold-labelled amyloplast (arrow) in a bean root tip, including labelling of starch grains (arrowheads). (B) Immunogold-labelled plastids (arrows) in a bean nodule. (C) Immunogold-labelled plastids (arrows) in a *Sesbania rostrata* root nodule. (D) Immunogold-labelled chloroplast (arrow) in a *S. rostrata* stem nodule. (E) Immunogold-labelled chloroplast (arrows) in an alfalfa leaf; note the relatively high-density labelling of the starch grains (arrowheads). (F) Plastids in a bean root tip treated with preimmune serum substituted for the γECS antibody (negative control). cyt, cytoplasm; ch, chloroplast; g, golgi; is, intercellular space; m, mitochondrion; n, nucleus; p, plastid; px, peroxisome; s, starch grain; v, vacuole. Bars, 1 μm.
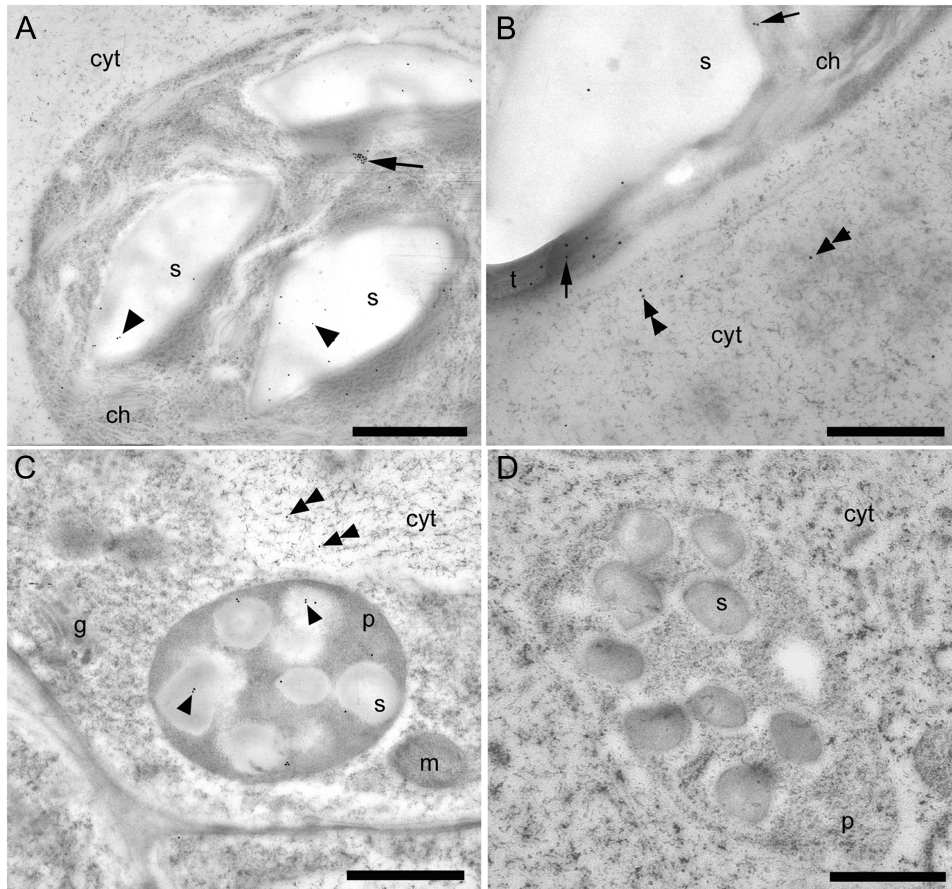
Fig. 3. Immunogold localization of homoglutathione synthetase (hGSHS) in leaves (A, B) and roots (C, D) of alfalfa. (A) Immunogold labelling of the interior of a chloroplast, which includes the chloroplast itself (arrow) and the starch grains (arrowheads). (B) Higher magnification of a chloroplast illustrating labelling of some of the thylakoids (arrows) and also sparse labelling in the adjacent cytoplasm (double arrowheads). (C) Amyloplasts in roots showing immunogold labelling of the starch grains (arrowheads) and very sparse labelling in the cytoplasm (double arrowheads). (D) Amyloplast in roots treated with preimmune serum substituted for the hGSHS antibody (negative control). cyt, cytoplasm; ch, chloroplast; g, golgi; m, mitochondrion; p, plastid; s, starch grain; t, thylakoids. Bars, 1 µm (A, C, D) and 500 nm (B).

current study found that γECS and *GSHS* were induced after 3 and 24 h of application of SNAP, whereas the *hGSHS* mRNA level remained unaffected (Supplementary Fig. S1, available at *JXB* online).

Effect of hormones on thiol synthetase transcripts and activities and on thiol contents of roots

A second type of experiment was performed to study regulatory mechanisms of the thiol biosynthetic pathway. Both non-nodulated and nodulated plants were used for comparison and the exposure time was prolonged to 48 h, so that the effects of hormones on the mRNA levels of thiol synthetase genes (Fig. 5A) could be reflected in the corresponding enzyme activities (Fig. 5B) and thiol contents (Fig. 5C) of the roots. However, the accurate quantification of GSH in roots required the use of HPLC-MS because GSH accounted for only ~3% of the total thiol tripeptides for both non-nodulated and nodulated plants. Furthermore, the extremely low GSHS levels precluded a reliable assessment of the effects of hormones on this enzyme activity in

the roots. The HPLC-MS method also served to confirm hGSH values obtained using HPLC-fluorescence. Both sets of data showed a high correlation ($r^2>0.90$, $n=40$–60) and therefore, for simplicity, only the hGSH contents obtained by HPLC-fluorescence are presented in Fig. 5.

The γECS mRNA level did not change after application of most hormonal treatments for 48 h. This gene was only slightly upregulated with ABA and downregulated with SA and PA in non-nodulated plants (Fig. 5A). By contrast, the expression of *GSHS* was markedly affected, particularly in non-nodulated plants. Notably, this gene was activated by CK and PA in both non-nodulated and nodulated plants, and was upregulated by JA and downregulated by ABA and SA in non-nodulated plants (Fig. 5A). The response of *hGSHS* was quite different, showing downregulation with CK and PA in non-nodulated plants and activation by IAA and downregulation by CK in nodulated plants (Fig. 5A). In roots of non-nodulated plants, the decreases of *hGSHS* mRNA levels with CK or PA were not accompanied by lower hGSHS activities (Fig. 5B). The same occurred in roots of nodulated plants treated with CK. In both types of
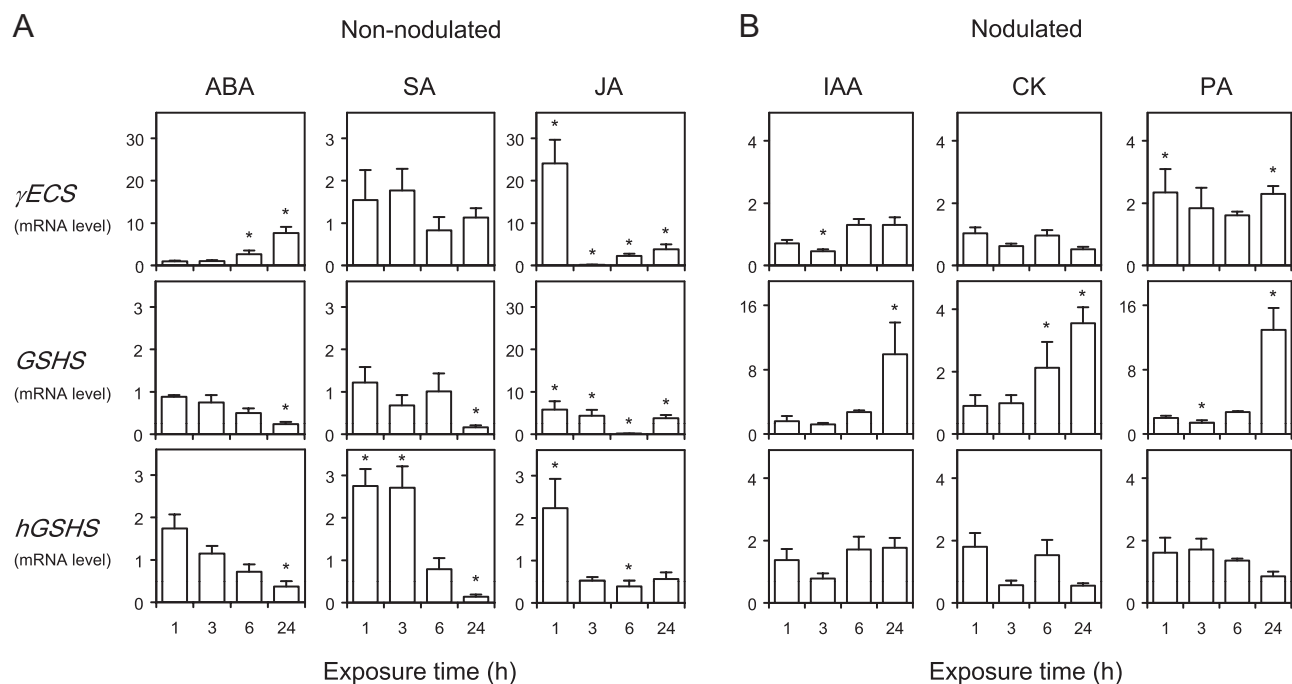
Fig. 4. Time-course patterns of expression of thiol synthetase genes in roots of *Lotus japonicus* in response to hormones. (A) Non-nodulated plants were supplied with 50 µM of abscisic acid (ABA), jasmonic acid (JA), or salicylic acid (SA) in the rooting medium. (B) Nodulated plants were supplied with 50 µM of indole-3-acetic acid (IAA), cytokinins (CK), or polyamines (PA) in the rooting medium. Steady-state mRNA levels of γ-glutamylcysteine synthetase (γECS), glutathione synthetase (GSHS), and homoglutathione synthetase (hGSHS) were normalized to *ubiquitin* and expressed relative to those of control plants. These were treated during the same time and with identical concentrations of NaOH (IAA and CK), ethanol (ABA, SA, and PA), or dimethylsulphoxide (JA) to those used to prepare the stock solutions of hormones. The mRNA levels of control plants were given a value of 1. All data are means ± SE of four or five replicates, corresponding to RNA extractions from different roots of two series of plants grown independently (two or three replicates per series). Asterisks denote upregulation (>2-fold) or downregulation (<0.5-fold) of the genes.

plants exposed to ABA, JA, or PA, there was a decrease in hGSH content despite no detectable variation in hGSHS activity, suggesting consumption or mobilization of the thiol in the roots (Fig. 5B, C).

Discussion

The subcellular localization of the GSH and hGSH biosynthetic pathway is an important aspect of thiol metabolism because these thiol tripeptides have multiple crucial functions and compartmentation of the enzymes would afford additional regulatory mechanisms in plants under physiological or stressful conditions (Bergmann and Rennenberg, 1993). The greater accuracy of immunolocalization has enabled the current study to clarify the previous contradictory reports on thiol localization. Early studies based on enzyme activity assays in isolated organelles led the authors to conclude that γECS, GSHS, and hGSHS are located in the plastids and cytosol (Klapheck *et al.*, 1988; Hell and Bergmann, 1990). Further work using reporter gene fusions and immunocytochemistry in *Arabidopsis* and Indian mustard, two members of the Brassicaceae family, indicated that γECS is confined to the plastids (Wachter *et al.*, 2005; Pasternak *et al.*, 2008). The immunogold labelling data presented here reveal that γECS is limited to plastids with no cytosolic localization (Fig. 2).

Interestingly, gold particles marking the presence of the γECS (Fig. 2A, E) and hGSHS (Fig. 3A, C) proteins were relatively abundant on the starch grains within the leaf chloroplasts and root amyloplasts. This localization strongly suggests a connection between (h)GSH biosynthesis and regulation of starch metabolism, possibly involving changes in redox-sensitive steps, and it is consistent with a proteomic study in which two enzymes involved in thiol synthesis, Cys synthase [*O*-acetylserine(thiol)lyase] and γECS, were detected in amyloplasts of wheat (*Triticum aestivum*) endosperm (Balmer *et al.*, 2006). A link between thiols and starch metabolism is also supported by the immunolocalization of glutathione peroxidase on starch grains in leaf chloroplasts and root and nodule plastids of *L. japonicus* and *S. rostrata* (Ramos *et al.*, 2009).

In this work, the hGSHS protein was found in the chloroplasts and root proplastids with lower amounts in the cytosol (Fig. 3), whereas in previous studies most or all hGSHS activity was detected in the cytosol (Klapheck *et al.*, 1988; Moran *et al.*, 2000). Because hGSHS is encoded by a single gene in legumes (Frendo *et al.*, 2001; Matamoros *et al.*, 2003), the cytosolic and plastidic isoforms derive from the same gene. In fact, both *GSHS* and *hGSHS* of *L. japonicus* contain sequences encoding potentially plastid transit peptides (Matamoros *et al.*, 2003). Therefore, the current results show that the final step of GSH and hGSH
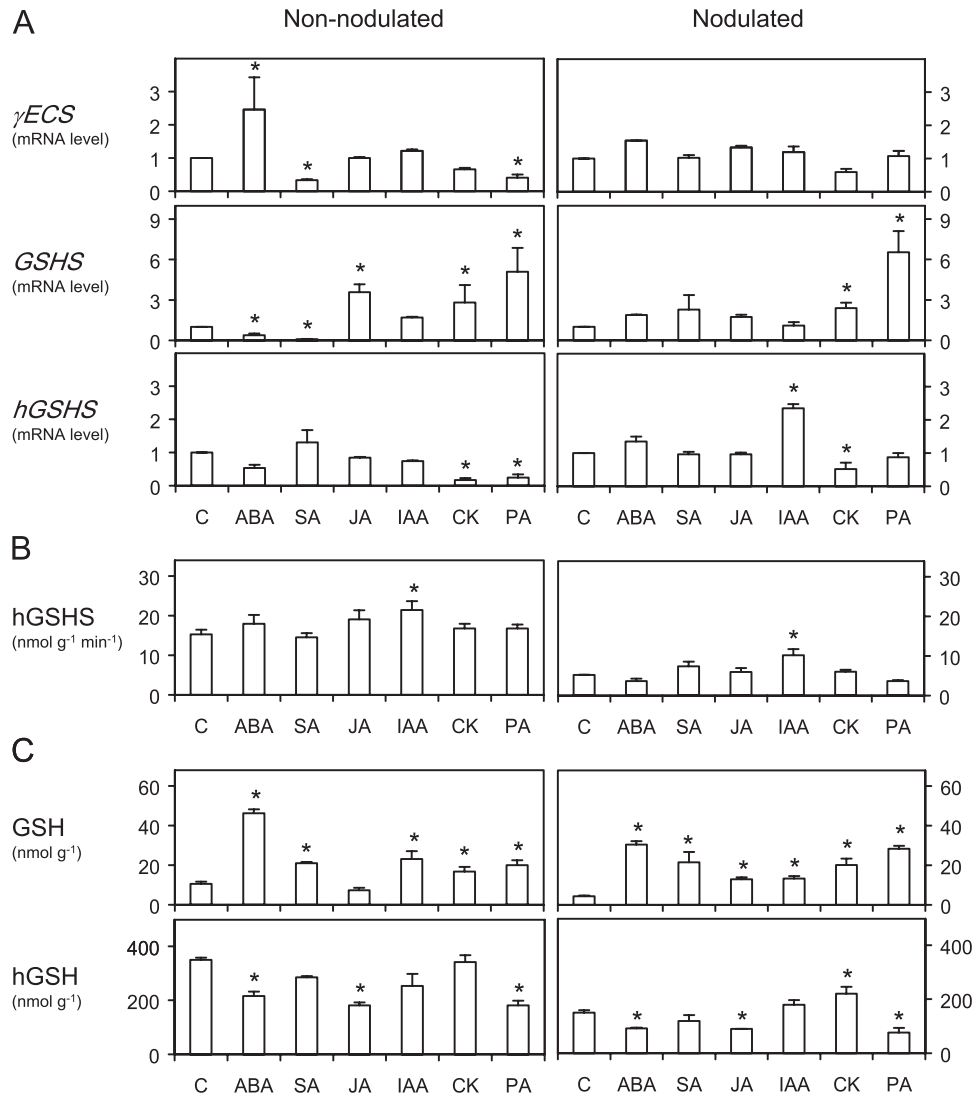
Fig. 5. Effects of hormones on the mRNA levels of thiol synthetase genes (A), homoglutathione synthetase (hGSHS) activity (B), and thiol contents (C) in roots of *Lotus japonicus*. No glutathione synthetase (GSHS) activity could be detected in any of the root extracts. Non-nodulated and nodulated plants were supplied for 48 h with 50 μM of hormones in the rooting medium. Steady-state mRNA levels of γ-glutamylcysteine synthetase (γECS), glutathione synthetase (GSHS), and homoglutathione synthetase (hGSHS) were normalized to *ubiquitin* mRNA levels and expressed relative to those of control plants (C). These were treated for 48 h with identical concentrations of NaOH (IAA and CK), ethanol (ABA, SA, and PA), or dimethylsulphoxide (JA) to those used to prepare the stock solutions of hormones. The mRNA levels of control plants were given a value of 1. Data of mRNA levels are means ± SE of four or five replicates, corresponding to RNA extractions of different roots from two series of plants grown independently (two or three replicates per series). Asterisks denote upregulation (>2-fold) or downregulation (<0.5-fold) of the genes. Values of thiol contents and enzyme activity of control plants were obtained from roots harvested immediately before the hormone treatments. Data of thiol contents and enzyme activity are means ± SE of four or six replicates, corresponding to extractions of different roots from two series of plants grown independently (two or three replicates per series). Asterisks denote that the means of the hormone treatments are significantly different from the control at $P < 0.05$ based on Student's *t*-test.

biosynthesis in legumes occurs in the plastids and cytosol, and that in both cases γ-glutamylcysteine is provided as substrate for GSHS and hGSHS by the chloroplasts in leaves and by the proplastids and amyloplasts in roots and nodules.

Two types of experiments were performed to examine in detail the regulatory mechanisms of thiol synthesis in response to hormones. As far as is known, such mechanisms have been investigated until now only for JA (Xiang and Oliver, 1998) and SA (Pucciariello *et al.*, 2009), probably because these compounds as well as GSH metabolism are directly associated with plant defence (Wingate *et al.*, 1988; Beckers and Spoel, 2006; Fujita *et al.*, 2006; Balbi and Devoto, 2008). However, previous reports have employed experimental approaches and plant systems different from those used here.

The strong upregulation of *γECS* and *GSHS* after exposure of non-nodulated plants of *L. japonicus* to JA for only 1 h (Fig. 4A) is fully consistent with the coordinated and rapid response of these genes to JA in *Arabidopsis* grown in soil or liquid cultures (Xiang and Oliver, 1998). This initial gene activation by JA in both model plants might be related to a function of GSH in their responses to biotic stress, as this thiol rapidly induces transcription of typical defence genes, such as phenylalanine ammonia-lyase and chalcone synthase (Wingate *et al.*, 1988). On the other hand, Pucciariello *et al.* (2009), using roots of *M. truncatula* deficient in (h)GSH, concluded that the thiol concentration modulates the SA-signalling pathway. In the present paper, it is shown that SA regulates, in turn, thiol biosynthesis. The antagonistic effects of SA and JA on the *GSHS* mRNA levels of non-nodulated roots of *L. japonicus* are obvious after 24–48 h, with an almost complete disappearance of the transcript after 48 h of SA treatment (Figs. 4A and 5A). Notably, these effects on mRNA levels were not accompanied by corresponding changes in the GSH content, which was even enhanced in the case of SA (Fig. 5C). Therefore, a post-transcriptional activation of GSHS and/or mobilization of GSH from leaves to roots may occur in plants after exogenous supply of SA. The post-transcriptional regulation of GSHS activity would provide a second controlling step of thiol biosynthesis, as the *γECS* activity of *Arabidopsis* is known to be regulated by redox changes of key Cys residues (Hicks *et al.*, 2007; Gromes *et al.*, 2008). By contrast, SA or JA did not affect hGSHS mRNA or activity levels (Fig. 5A, B), indicating a completely independent regulation of *GSHS* and *hGSHS* and, probably, of the enzyme activities.

This differential regulation was further underscored by another novel observation. The application of IAA for 24 h to nodulated plants caused a strong activation of *GSHS* but had no effect on the other two genes (Fig. 4B). After 48 h of treatment, IAA was the only hormone eliciting changes in hGSHS activity in the roots of both non-nodulated and nodulated plants (Fig. 5B). This auxin also caused induction of *hGSHS* in nodulated plants but no change in the hGSH content (Fig. 5C). These results strongly suggest that GSH and hGSH are not functionally equivalent and that, at least in nodulated plants, the regulation of hGSHS activity by auxin occurs at the transcriptional level.

Interestingly, two 'classic' hormones that play key roles in cell division, namely CK (Dello Ioio *et al.*, 2008) and PA (Bouchereau *et al.*, 1999; Theiss *et al.*, 2002), upregulated *GSHS* after 24–48 h but either did not change or decreased *hGSHS* mRNA levels (Figs. 4B and 5A), lending credence to a specific function of GSH in this process. Furthermore, the activation of *GSHS* was accompanied by an increase of GSH in the roots, whereas hGSH remained almost unchanged with CK and decreased with PA (Fig. 5C). The different responses of *GSHS* and *hGSHS* cannot be interpreted in terms of a functional compensation between the corresponding thiols because the concentration of GSH in roots and leaves is far too low compared to that of hGSH. The expression pattern of thiol synthetases of roots of nodulated plants supplied for 24 h with PA (Fig. 4B) was similar to that elicited with NO donors after 3–24 h (Supplementary Fig. S1), namely, transcriptional activation of *γECS* and *GSHS* but not of *hGSHS*. This suggests that NO is mediating the effects of PA, consistent with recent reports showing that PA may directly or indirectly regulate NO synthesis (Tun *et al.*, 2006; Yamasaki and Cohen, 2006). These results are in keeping with those reported for *M. truncatula* roots treated with the NO donors sodium nitroprusside and nitrosoglutathione (Innocenti *et al.*, 2007), and indicate that the differential regulation of *GSHS* and *hGSHS* by NO is probably widespread in legumes. The contrasting response of the two genes to NO, which is a crucial signalling molecule in plants and other organisms, is probably biologically relevant and further supports the hypothesis that GSHS and hGSHS play different roles in legumes. This is somewhat surprising taking into account that *GSHS* and *hGSHS* display high sequence homology and have originated by duplication in both *M. truncatula* (Frendo *et al.*, 2001) and *L. japonicus* (Matamoros *et al.*, 2003).

Contrary to the positive relationship between *GSHS* transcript levels and GSH contents observed with CK and PA, the two parameters were inversely related in non-nodulated plants treated with ABA (Fig. 5A, C). The increase of GSH in plants following exposure to ABA is difficult to explain in the absence of measurements of γECS and GSHS activities, which could not be assayed because of the lability and low abundance of the enzymes. Another complicating factor was the possible post-translational regulation of γECS by the redox environment (Hicks *et al.*, 2007; Gromes *et al.*, 2008), which may vary with the hormonal treatment. However, the fact that GSH was also increased in nodulated plants with ABA, without any change in γECS and *GSHS* mRNA levels, indicates a post-transcriptional control of the enzyme activities and/or mobilization or lower consumption of GSH in these plants, as mentioned for SA.

In conclusion, the results demonstrate the existence of subcellular compartmentation of the thiol biosynthetic pathway in legume leaves, roots, and nodules. They also reveal a selective regulation of the three thiol synthetase genes by hormones and NO. Notably, *GSHS* and *hGSHS* are differentially regulated by most hormones examined. The contrasting response of the two genes and the two thiols to hormones suggests distinct functions for GSH and hGSH in cell division, organ development, and stress signalling. Moreover, with a few exceptions, the response profiles of the GSH and hGSH contents to the various hormonal treatments are similar in the roots of non-nodulated and nodulated plants, indicating that thiol homeostasis is independent of the nitrogen source.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Effect of NO on the expression of γ-glutamylcysteine synthetase (γECS), glutathione synthetase (GSHS), and homoglutathione synthetase (hGSHS) genes in roots of *Lotus japonicus*.

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