

Effects of phosphinotricin treatment on glutamine synthetase isoforms in Scots pine seedlings

Concepción Avila, Angel García-Gutiérrez, Remedios Crespillo, Francisco M. Cánovas*

Laboratorio de Bioquímica y Biología Molecular, Facultad de Ciencias-Instituto Andaluz de Biotecnología, Universidad de Málaga, Campus de Teatinos, 29071 Málaga, Spain.

* Author to whom correspondence should be addressed (fax +34 5 2132000; e-mail canovas@uma.es)

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Abstract – The occurrence of GS isoenzymes has been investigated in Scots pine (*Pinus sylvestris*) seedlings. A transient increase of glutamine synthetase (GS, EC 6.3.1.2) activity was observed in the cotyledon whorl of plants treated with the herbicide phosphinotricin (PPT). The increase in GS activity was accompanied by a parallel accumulation of GS1 protein, which remained at high levels throughout the PPT treatment. Two-dimensional SDS-PAGE western analysis showed that pine extracts contained two GS1 polypeptides which differ in their corresponding isoelectric points. Analysis of crude extracts by ion-exchange chromatography led to the separation of two GS isoforms. The first peak (GS1-a) eluted from the columns at a low ionic strength (0.15–0.18 M KCl), whereas the second one (GS1-b) was detected at 0.5 M KCl. A detailed molecular study of both GS holoenzymes confirmed that their subunits were similar in size (about 41 kDa) but different in charge. All these data clearly demonstrate the presence of two GS1 forms in Scots pine cotyledons. Moreover, a comparison of isolated GS isoproteins with the recombinantly expressed Scots pine cytosolic subunit suggests that GS1-a corresponds to the previously characterized cDNA (pGSP114) whereas GS1-b is a minor GS isoenzyme with increased relative abundance in phosphinotricin treated plants. © Elsevier, Paris

2D-Electrophoresis / glutamine synthetase / isoenzymes / phosphinotricin / *Pinus sylvestris*

2-D, two dimensional / DEAE, diethylaminoethyl / GS, glutamine synthetase / IEF, isoelectrofocusing / PAGE, polyacrylamide gel electrophoresis / pI, isoelectric point / PPT, phosphinotricin / SDS, sodium dodecylsulphate

1. INTRODUCTION

In higher plants, glutamine synthetase (L-glutamate ammonia ligase, ADP-forming, EC 6.3.1.2) (GS) is a key enzyme in ammonia assimilation [18]. Native GS is an octameric enzyme with a molecular size of about 330–380 kDa that generally exists as two major groups of isoforms: GS1 localized in the cytosol and GS2 confined to the plastid [19]. GS isoenzymes have been purified and characterized from many plant species [18]. Progress in the last few years demonstrated that the GS isoforms exist as a small family of nuclear genes which are differentially regulated depending on light conditions or developing state [8, 17]. The complete nucleotide sequences of chloroplastic and cytosolic GS subunits have been deduced from the corresponding cDNAs isolated from a variety of angiosperm species [11].

The available information about GS in woody plants and particularly in gymnosperms is much more limited. The enzyme has been purified from needles and roots of jack pine and Douglas fir roots, and its physico-chemical and kinetic properties determined [1, 26]. Current data regarding the subcellular localization of glutamine biosynthesis indicate that seedlings of pine and other conifer species mainly express cytosolic GS in photosynthetic tissues [5, 13]. GS2 isoenzyme and its corresponding polypeptide was not detected in maritime [4] and Scot pines [5, 6] by biochemical, immunological and molecular approaches. Molecular data derived from the characterization of GS cDNA clones in several laboratories [5, 10, 15] shows that the GS1 gene is actively expressed in developing pine seedlings. GS2 gene expression has not yet been demonstrated in a reliable way. Taken together, the above data indicate that glutamine bio-

synthesis occurs in the cytosol of pine cells, at least during the early phases of plant growth. This molecular-based assumption has been recently confirmed by the immunocytochemical detection of GS1 in mesophyll and phloem cells of pine seedlings [13], suggesting a key role for cytosolic GS in the early development of conifers. However, it remains to be determined whether the same or different gene products (GS1) are present in both cell types. In this paper, we report the existence of two GS isoforms in Scots pine. An additional GS isoform has been identified with different chromatographic behaviour and subunit composition but of similar size compared to the previously characterized cytosolic enzyme.

2. RESULTS

2.1. GS accumulation in response to PPT

Scots pine seedlings were sprayed twice with a 25-mM phosphinotricin solution at a 3-h interval between treatments. Cotyledon samples were then taken at 8, 24 and 48 h after treatment, protein extracted and GS activity determined by the transferase assay (table I).

Table I. Changes in GS activity determined in Scots pine cotyledons following phosphinotricin treatment. Seedlings with a cotyledon size range of 20–25 mm (about 15 d after imbibition) were sprayed with the herbicide and GS activity determined by the transferase assay at the indicated times: 8, 24 and 48 h after phosphinotricin application.

Sample	GS activity	
	(nkat·g ⁻¹ FW)	(nkat·mg ⁻¹ protein)
Untreated	921	134
Phosphinotricin		
8 h	4 710	509
24 h	991	110
48 h	997	116

GS activity increased dramatically (about 4–5-fold) after 8 h of PPT treatment when compared with the levels detected in control plants. However, this increase was only transitory and enzyme activity declined to the control values 24 and 48 h post-herbicide application. Similar results are obtained when GS activity is expressed in a fresh weight or a total protein basis, indicating that observed changes are due to variations in GS activity levels and not to a modification of total protein content triggered by PPT. In order to check if the increase in GS activity can be explained

by enzyme activation or if it was correlated to an increase in GS protein, the same samples were analyzed by western blot using antibodies raised against the pine recombinant GS expressed in *Escherichia coli* [6]. Figure 1 shows that a major polypeptide of about 41 kDa was detected under all conditions tested. A minor faster-migrating band of about 28 kDa was also apparent, but was considered to be a degradation product of the intact polypeptide. No other GS polypeptides of greater size were present on the blots. Interestingly, the relative abundance of the 41-kDa band increased in PPT-treated plants, but in contrast with GS activity, its steady state level remained unaltered throughout the period of herbicide treatment. These findings demonstrate that GS activity enhancement observed in response to PPT was concomitantly accompanied by a parallel accumulation of GS protein.

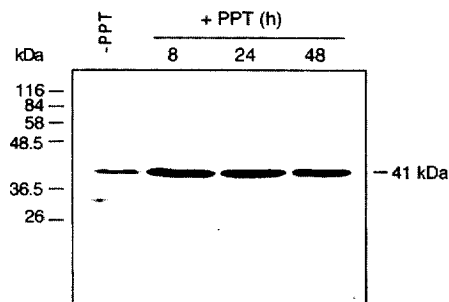


Figure 1. Immunoblot analysis of GS protein in Scots pine cotyledons following phosphinotricin (PPT) treatment. The same protein extracts in which the GS activity was determined (table I) were subjected to SDS-PAGE, the resolved polypeptides electrotransferred to nitrocellulose membranes and GS protein immunorevealed using specific antibodies raised against pine recombinant GS. A dilution of purified antiserum 1:9 000 was used. Thirty micrograms of proteins were loaded per well.

To investigate the possibility that the observed GS accumulation was a general response found in the whole plant, enzyme activity was also measured in hypocotyls and roots during the time course of PPT-treatment. No significant changes were apparent in these tissues (data not shown), indicating that the short-term effect of PPT appears to be restricted to green cotyledons and hence, this tissue was used as the source of plant material for subsequent experiments.

2.2. Separation of GS isoenzymes

To get further insights on this physiological response in molecular terms, it was interesting to determine

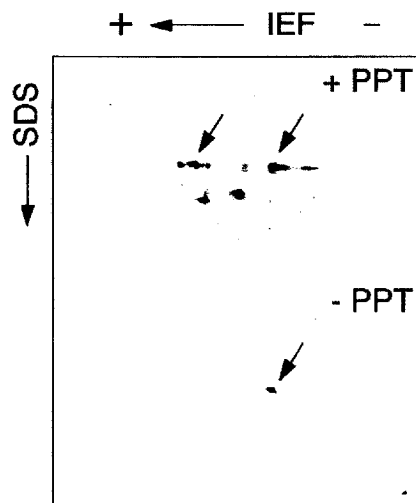


Figure 2. Separation of GS polypeptides in Scots pine crude extracts by two-dimensional western blot analysis. Total proteins (50 μ g) were subjected to electrofocusing (first dimension gel with a pH range of 3–10) and SDS-PAGE (12.5% resolving gel). Separated polypeptides were blotted onto nitrocellulose filters and probed with the anti-GS antibody. Results derived from 8-h phosphinotricin treated plants (+ PPT), and from untreated plants' (- PPT) control pine seedlings grown without the herbicide. The position of GS polypeptides is indicated by arrows.

whether or not more than one immunoreactive species (as co-migrating bands) were present in the accumulated GS polypeptide. To check this possibility, protein extracts were subjected to two-dimensional SDS-PAGE gels, polypeptides electrotransferred to nitrocellulose filters and immunoprobed with GS antibodies (figure 2). As shown in figure 2 + PPT, the 41-kDa band was resolved as two major bands (arrows) of similar size but different charge in PPT-treated plants. Other faster-migrating spots were assumed to be degradation products of intact GS subunits because they were not detected in purified protein extracts. In contrast, only one major spot was apparent in cotyledons from untreated control plants when analyzed by two-dimensional SDS-PAGE (figure 2 - PPT). These results suggest the appearance of an additional GS subunit in response to PPT treatment. The above results prompted us to examine the presence of GS isoenzymes in the cotyledons of PPT-sprayed pine seedlings by anion-exchange chromatography on DEAE-Sephacel. Figure 3 A (+ PPT) shows that GS activity eluted in two peaks, the first one (GS1-a) at 0.15–0.18 M KCl and a second smaller peak (GS1-b) eluting from the column at 0.5 M KCl. However, GS1-a was largely the major GS isoform in the cotyledons of untreated control plants (figure 3 A, - PPT).

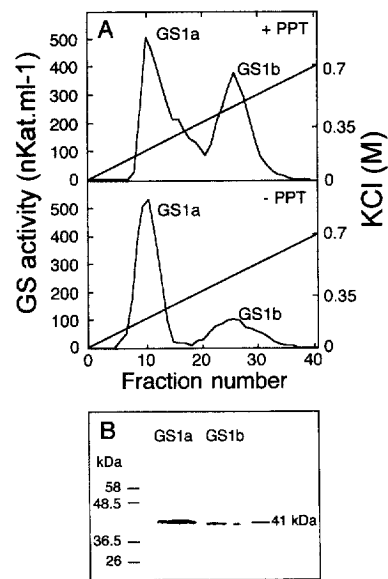


Figure 3. Elution profile of GS activity from DEAE-Sephacel and immunoblot analysis of GS isoenzymes. **A**, Crude extracts from cotyledons of 8-h phosphinotricin-treated (+ PPT) or untreated (- PPT) plants were fractionated by ammonium sulphate, dialyzed and loaded onto a DEAE-Sephacel column. Proteins were separated by ion exchange chromatography and enzyme activity determined by the transférse assay. The GS elution profiles are representative of at least 2 independent experiments. **B**, Western blot analysis of peaks GS1-a and GS1-b. Active fractions for GS1-a (fractions number 10–15) and for GS1-b (fractions number 25–30), were pooled separately, concentrated and tested for the presence of GS protein by western blot analysis. The PPT-GS profile was selected for protein analysis. In each case, 10 μ g of proteins from each peak were loaded per well.

2.3. Subunit composition of GS isoenzymes

In order to determine the subunit composition of these two GS holoenzymes, fractions exhibiting high level activity from both peaks were collected separately and tested by western blot analysis using pine anti-GS antibodies. Figure 3 B shows that a single band of about 41 kDa was present in the two GS peaks and it can, therefore, be concluded that GS isoenzymes are made up of subunits of similar size. Following the separation of the two GS isoenzymes by DEAE-Sephacel chromatography, their subunit composition was also studied by two-dimensional gel electrophoresis (2-D PAGE) and western blotting (figure 4 A). Immunostaining of the 2-D gels showed that GS1-a holoenzyme was made up of a subunit with an estimated pI of 6.5 whereas the calculated value for the GS1-b subunit was about 5.6 (figure 4 A, compare GS1-a and GS1-b). These results indicate that GS

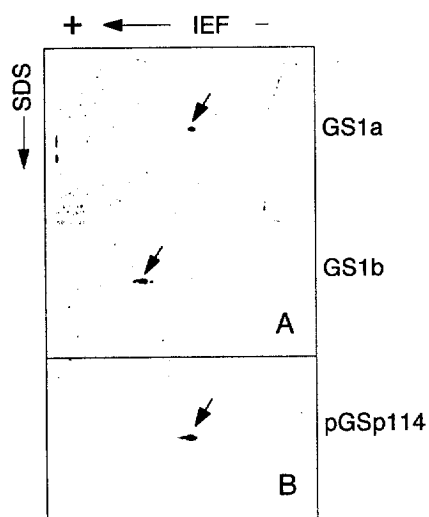


Figure 4. GS subunit composition of the isolated GS isoenzymes. **A**, DEAE-Sephacel pooled GS active peaks GS1-a and GS1-b were analyzed by two-dimensional western blot analysis as described in the legend of figure 2. The position of GS subunits is indicated by arrows (GS1-a and GS1-b). **B**, A cDNA clone (pGSP114) encoding a Scots pine cytosolic subunit [5] was subcloned in the expression vector pET3c to overproduce the GS polypeptide in *Escherichia coli* cells [6]. The recombinant protein was subjected to two-dimensional western blot analysis as described in the legend of figure 2. The position of the pGSP114 protein product is indicated by an arrow. In all cases, 10 μ g of protein were loaded per gel.

isoenzymes are composed of different subunits in perfect agreement with the different ionic strength at which they eluted from the DEAE-Sephacel column (figure 3 A). Moreover, it should be noted that the full length cDNA clone reported by Cantón et al. [5] encodes a GS1 polypeptide with a theoretical isoelectric point of 6.6 which corresponds to that of the GS1-a subunit. This finding suggests a close similarity between the previously characterized GS1 enzyme and GS1-a. In order to confirm this possibility, the recombinant protein for this cDNA clone (pGSP114) [6] was analyzed in the same experimental conditions by 2-D PAGE and immunoblotting (figure 4 B). The electrophoretic behaviour and isoelectric point calculated for this protein is similar to the one obtained for the GS1-a isoenzyme (figure 4, compare GS1-a and pGSP114).

2.4. Northern hybridization

The availability of a cDNA clone encoding GS1 [5] prompted us to examine the effect of PPT treatment on GS1 expression at the mRNA level by hybridization with a specific probe. As shown in figure 5, the steady-

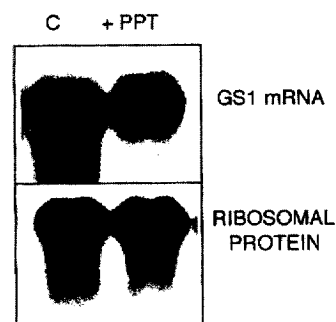


Figure 5. Northern blot analysis of GS1 mRNA in the cotyledons of Scots pine seedlings. Total RNAs extracted from control untreated (C) and 8-h phosphinotricin-treated plants (+PPT) were fractionated through 1% formaldehyde agarose gels, and probed with 32 P-labelled cDNA insert of clone pGSP114 [5]. The loading of equivalent amounts of RNA (10 μ g) in each lane was normalized by probing the same filters with a ribosomal protein probe (RBP4).

state level of GS1 mRNA in green cotyledons of Scots pine seedlings did not increase in the presence of PPT with regard to control plants, suggesting no induction of gene expression corresponding to pGSP114.

3. DISCUSSION

It is now well established that GS1 isoenzyme is the predominant GS isoform in conifer seedlings ([4, 6, 13] and this work). Nevertheless, data derived from Southern blotting analysis of pine genomic DNA suggested that GS is encoded by a gene family [5, 15], as in several angiosperms [14, 24]. Very recently, it has been reported that the herbicide phosphinotricin, a known blocking agent of GS activity, induces changes in the expression of GS genes [20, 22, 23]. Moreover, it has also been reported that another GS inhibitor (methionine sulfoximine) induces the accumulation of GS1 polypeptides in tobacco cell cultures [30].

In an attempt to identify new GS isoproteins in pine, we have investigated protein and enzymatic activity following the application of PPT to developing seedlings. Our results indicate that the herbicide triggers the accumulation of GS protein with a peak in GS activity observed at 8 h of treatment (table 1). To our knowledge, this short-term increase in GS abundance has not been reported previously, and it can be concluded that either the effect of PPT-inhibition was so early and transient that it was all over before the first time point (at 8 h) or PPT did not inhibit GS at the concentration used and the effect is exerted in some other way. Two dimensional gel electrophoresis and western blot analysis demonstrate that PPT-induced

GS accumulation is associated with the synthesis of at least one additional GS polypeptide in pine seedlings (*figures 1 and 2*). The presence of PPT also triggered the appearance of a GS1 polypeptide in tomato cotyledons and leaves [20, 22] although no molecular explanation was found. Our data indicate that GS accumulation is not a general response in the whole plant but is restricted to green cotyledons suggesting a dependence on photosynthetic metabolism. These findings are consistent with the recent work by Pérez-García et al. [23] reporting a close correlation between the photosynthetic process and changes in the relative proportions of GS isoforms in tomato leaves. In fact, Wild et al. [29] demonstrated that herbicide application provokes inhibition of photosynthesis. However, the understanding of phosphinotricin metabolic effects on nitrogen metabolism is a complex task, because the herbicide causes not only ammonium accumulation but also a great decrease in amino donors [28, 29] and therefore a C/N imbalance. Whether these factors are involved in the stimulation of GS1 expression remains to be determined. In contrast, it is known that the long-term increase in GS activity reported in PPT-resistant alfalfa cell lines [7] was due to a gene amplification phenomenon.

The observed short-term increase in GS activity allowed the separation by ion-exchange chromatography of two active GS isoenzymes, GS1-a and GS1-b, and their further characterization (*figures 3 and 4*). GS1-a eluted from the columns at low ionic strength, as does the major GS isoenzyme previously reported in pine seedlings [4, 27]. Furthermore, we have also demonstrated that the GS1-a subunit has a charge similar to that of the recombinant product of Scots pine GS1 cDNA [6]. In contrast, GS1-b was eluted from the ion-exchange column at a higher salt concentration and probing showed it to be constituted of a GS subunit with a different charge (*figure 4*). We have also determined that this GS isoform is present, in lower abundance, in control pine cotyledons (*figure 3 A*). This molecular form may represent an extensively modified subunit derived from the first one, or a new type, product of an alternative GS1 gene. In support of the latter possibility, we have found no stimulation of GS1 mRNA abundance in response to PPT when expression was examined using pGSP114 as a probe (*figure 5*).

In summary, results shown in this work strongly suggest that GS1-a corresponds to the major GS isoenzyme detected in Scots pine cotyledons [5, 9] whereas GS1-b is a minor enzyme whose relative

amount is enhanced by the PPT treatment. Although the subcellular localization of this novel GS isoform has not been determined in this study, our results suggest that GS1-b is a cytosolic enzyme. The existence of distinct, organ-specific and developmentally regulated isoenzymes of GS in higher plants is already well-known. However, the existence of GS1-b in cotyledons of pine and its metabolic role in ammonia assimilation by the seedlings remain to be determined, considering the fact that GS1-a is much more abundant and its level is sufficient to maintain the plant nitrogen economy.

Work is in progress in our laboratory to characterize the physiological role of this protein in cotyledons and other tissues in the plant, such as needles, roots or stems. The eventual isolation of a cDNA sequence encoding this protein will determine whether or not GS1 isoenzymes are encoded by separate genes in pine.

4. METHODS

4.1. Chemicals. All chemicals were of analytical grade. Biochemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise specified. All SDS-PAGE material was from Boehringer Mannheim GmbH (Mannheim, Germany). The carrier ampholytes pH 3–10 for 2-D PAGE were pharmalytes from Sigma Chemical Co. Immunochemical kits were from Vector labs (Burlingame, CA, USA).

4.2. Plant material and growth conditions. The Scots pine seeds (*Pinus sylvestris* L.) used in all experiments were from the Servicio de Material Genético, ICONA (Instituto de Conservación de la Naturaleza), Estación 'El Serranillo', Guadalajara (Spain). The seeds were aerated in distilled water for 2 days, then transferred to plastic pots containing peat. Germinated seedlings were grown at a 16-h light/8-h dark regime at 24 °C in a controlled growth chamber (Koxka, Spain). Illumination was provided by fluorescent lights (150 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). No external nitrogen was added to the plants. Seedlings with cotyledons of 2.0–2.5 cm in length were used for the treatment with a 25-mM phosphinotricin solution containing 0.02 % (v/v) Tween-20. The herbicide was sprayed over the plants and samples were taken 8, 24 and 48 h after the treatment.

4.3. Enzyme extraction. Five to 10 g of frozen cotyledon tissue were pulverized in a pre-chilled pestle and mortar using liquid nitrogen. Then, 4–5 volumes of an extraction medium consisting of 50 mM Tris (pH 8.0), 1 mM MnCl_2 and 3.5 mM β -mercaptoethanol were added. The homogenate was squeezed through 4 layers of cheesecloth and centrifuged at $22\,000 \times g$ for 30 min at 4 °C.

4.4. Ammonium sulphate fractionation. The crude extract was brought to 20 % saturation $(\text{NH}_4)_2\text{SO}_4$ and the precipitate was removed by centrifugation at $22\,000 \times g$ for 30 min. Then, the supernatant was brought to 80 % saturation of $(\text{NH}_4)_2\text{SO}_4$. The solution was centrifuged, and the precipitate dissolved in 2 mL of the same buffer and dialyzed overnight against 2 L of the same buffer. All procedures were carried out at 4 °C.

4.5. Anion-exchange column chromatography. Glutamine synthetase isoenzymes were separated as described earlier [12] with some modifications. The desalted protein extract was loaded on a DEAE-Sephacel column (12 × 100 mm) pre-equilibrated with buffer. The column was washed with 50 mL of buffer and GS was eluted by a 60-mL linear KCl gradient (0–0.7 M). Fractions of approximately 1.5 mL were collected at $20 \text{ mL} \cdot \text{h}^{-1}$ and used to determine GS activity. Those with activity were pooled and used for SDS-PAGE, western blot and 2-D PAGE.

4.6. SDS-PAGE and western blot analysis. Proteins were analyzed by denaturing gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) following the system described by Laemmli [16]. The stacking gel was 5 % (w/v) polyacrylamide and the separating gel was 12.5 % (w/v). Resolved polypeptides were electrotransferred onto nitrocellulose membranes using a MultiphorII Novablot apparatus (LKB, Uppsala, Sweden) following the method described by Towbin et al. [25]. Immunodetection of GS polypeptides was carried out exactly as described in Cánovas et al. [4]. Prestained protein markers were used to determine the molecular size of GS subunits on the filters: β -galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), triosephosphate isomerase (26 kDa).

4.7. Two dimensional electrophoresis of proteins. Two-dimensional gel electrophoresis was carried out using O'Farrell's technique [21] with minor modifications. The isoelectrofocusing (IEF) slab gels were $80 \times 70 \times 0.75 \text{ mm}$ of a mixture consisting of 4 % acrylamide, 9.2 M urea, 2.5 % carrier ampholites (Pharmalyte pH 3–10). The IEF was performed at 200 V for 2.5 h. The second dimension electrophoresis was also performed in $80 \times 70 \times 1 \text{ mm}$ slab gels of 12.5 % (w/v) polyacrylamide gels containing SDS as described above. The gels were then transferred to nitrocellulose membranes and immunodetection was performed essentially as described for one-dimensional western blots.

4.8. Protein and glutamine synthetase activity determination. Protein contents were determined by Bradford's method using bovine serum albumin as a standard [2]. Glutamine synthetase activity (L-glutamate ammonia ligase, ADP-forming, EC 6.3.1.2) was measured using the transferase assay exactly as described by Cánovas et al. [3].

4.9. Northern analysis. Total RNA was prepared by homogenizing pine cotyledons as described earlier [5], fractionated on a 1.0 % (w/v) agarose gel, 0.66 M formaldehyde in MOPS (N-morpholino propane sulfonic acid) buffer, trans-

ferred to Nytran filters (Schleicher-Schüell) and hybridized with a radioactively labelled (^{32}P) pGSP114 cDNA insert as a probe. Hybridizations were carried out at 42 °C in 50 % (v/v) formamide, 5× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH_2PO_4 , 1 mM EDTA) 5× Denhardt's, 0.1 % (w/v) SDS and $100 \text{ mg} \cdot \text{mL}^{-1}$ salmon sperm DNA. Filters were washed twice in 6× SSPE/0.1 % SDS at room temperature and 1× SSPE/0.1 % SDS at 65 °C.

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