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ORIGINAL ARTICLE

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Molecular analysis of the 5′-upstream region of a gibberellin-inducible cytosolic glutamine synthetase gene (*GS1b*) expressed in pine vascular tissue

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Abstract The promoter region of the cytosolic glutamine synthetase *GS1b* gene was isolated from the conifer *Pinus sylvestris* L. (Scots pine). The 1,171-bp stretch of sequence lying immediately upstream of the transcriptional start site was sufficient to drive the expression of a reporter gene in a manner consistent with the expression pattern of the native *GS1b* gene. Computer analysis of putative *cis* elements in this promoter region revealed the presence of an AT box, an AC motif similar to those found in other genes expressed in the vascular tissue, and a gibberellin (GA)-responsive element. Consistent with the latter finding, *GS1b* gene expression was induced by exogenously supplied gibberellic acid (GA_3) in germinating pine embryos and pine seedlings. In order to examine if the putative GA-response element found in the *GS1b* promoter could function in the regulation of *GS1b* expression, a series of deletions of the upstream gene region were fused to the *uidA* reporter gene, and transient expression analyzed either in untreated or in GA_3-treated pine (*Pinus pinaster* Ait.) protoplasts. Deletion analysis revealed that sequences containing the GA-responsive element, located between −1005 and −724 bp were essential for the increased promoter activity observed in response to GA_3. Furthermore, electrophoretic-mobility-shift assays showed that pine nuclear proteins bind to a 22-bp sequence that contains the GA-response element, located between −768 and −747 bp relative to the transcription start site.

Keywords Ammonium assimilation · *cis* elements · Cytosolic glutamine synthetase · Gibberellic acid · *Pinus*

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Abbreviations *ABA* Abscisic acid · *BAP* N^6-Benzylaminopurine · *DAI* Days after imbibition · *GA* Gibberellin · *GA_3* Gibberellic acid · *GS* Glutamine synthetase · *GUS* β-Glucuronidase · *IAA* Indole-3-acetic acid · *TDZ* Thiadiazuron

Introduction

Glutamine synthetase (GS, EC 6.3.1.2) is a crucial enzyme in ammonium assimilation in higher plants. The metabolic requirement for GS activity in ammonium assimilation in plants is fulfilled by different GS isoforms expressed in specific organs and at specific developmental stages (Cren and Hirel 1999; Ireland and Lea 1999). Two different classes of GS have been reported in angiosperms, and each is found in a different subcellular compartment. The GS1 class is found in the cytosol; GS2 is localized to the chloroplast.

In angiosperms, GS2 is the predominant isoform found in photosynthetic leaf mesophyll cells, where it plays a major role in the assimilation of ammonium derived from nitrate reduction and photorespiration (Lam et al. 1996). In contrast, the cytosolic GS1 is the predominant isoform in roots and other non-photosynthetic tissues. While GS2 is encoded by a single nuclear gene, GS1 is encoded by a small gene family whose members are expressed differentially during development or in response to different external stimuli, to give rise to a variable number of GS1 isoforms (Ireland and Lea 1999). The physiological roles of the different GS1 isoforms are unclear but recent work suggests that GS1 could be involved in a range of processes including the primary assimilation of ammonium from the soil (Sakakibara et al. 1996), N recycling and translocation between source and sink tissues, or the re-assimilation of N mobilized during senescence (Cren and Hirel 1999).

Since ammonium assimilation is a crucial process in plants, the molecular characteristics of GS have been extensively studied in herbaceous plants. In contrast,

much less is known about the characteristics of GS in woody plants, particularly gymnosperms, despite their economic and ecological importance. Woody perennial plants are a unique model with which to study ammonium assimilation since, in many woody species, N assimilated from the soil is not used immediately but is stored in the bark to be further remobilized the next growing season for the formation of new developing organs (Coleman et al. 1994).

In gymnosperms, GS has been purified from needles and roots of jack pine (*Pinus baksiana*) and from roots of Douglas fir (*Pseudotsuga menziesii*), and its physicochemical and kinetic properties have been determined (Vézina and Margolis 1990; Bedell et al. 1995). With regard to the subcellular localization of the enzyme, in the photosynthetic tissues of pine seedlings and other conifers only cytosolic isoforms of GS (GS1) have been identified. The chloroplast isoform (GS2) has not yet been detected in conifers despite using a number of different molecular approaches (Cánovas et al. 1998). The localization of the GS protein exclusively in the cytosol of photosynthetic and non-photosynthetic pine cells was demonstrated by immunocytolocalization (García-Gutiérrez et al. 1998). These data indicated that glutamine biosynthesis occurs in the cytosol of pine cells, not only during the initial stages of pine development but also mature pine trees (Avila et al. 2000).

In cotyledons of *Pinus sylvestris* (Scots pine), two GS1 isoforms, GS1a and GS1b, have been reported (Cantón et al. 1993; Avila et al. 1998). The pine GS1 isoforms are composed of subunits of a similar size, but different charge. They have overlapping but distinct patterns of expression that differ throughout development, and by cell type (Avila et al. 2001a). In pine seedlings, expression of GS1a is restricted to tissues containing chloroplasts and the expression in these tissues is strongly stimulated by light (Cantón et al. 1999). These data support a role for GS1a in the generation of amino donors for the biosynthesis of major N compounds in photosynthetic tissues, a role similar to GS2 in angiosperms. By contrast GS1b is most abundant in hypocotyls and roots, although it is also present at a low level in the cotyledons. In all these tissues, *GS1b* expression is associated with vascular bundles (Avila et al. 2001a). This pattern of expression is quite similar to that found for GS1 in angiosperms (Dubois et al. 1996) and suggests that GS1b plays an important role in N transport and translocation within the seedling. However, other functional roles for this isoform in tree biology cannot be ruled out.

Since there is no available information about transcriptional regulation of *GS* genes in conifers, our laboratory is interested in the isolation and functional characterization of pine *GS1* promoters. In previous work (Avila et al. 2001b) the ability of the *GS1a* promoter to drive gene expression in pine cotyledons and transgenic *Arabidopsis* was reported. The 5′-untranslated region of the gene contained regulatory sequences involved in the transcriptional activation by light. Furthermore, we have identified the presence of putative *cis* elements and characterized how they interact in the transcriptional regulation of the gene (data not shown).

The present work examines the promoter region of the *GS1b* gene to identify regulatory elements that could underpin the expression of *GS1b*.

Materials and methods

Plant material

Maritime pine (*Pinus pinaster* Ait.) and Scots pine (*Pinus sylvestris* L.) seeds used in all experiments were from Servicio de Material Genético, Instituto de Conservación de la Naturaleza, Madrid, Spain. Seed germination and growth of the seedling were as described previously (Cánovas et al. 1991).

Isolation of promoter DNA

The *GS1b* promoter from *P. sylvestris* was amplified by PCR walking using the protocol described by Devic et al. (1997). Approximately 5 μg of genomic DNA was digested overnight with 160 U of each enzyme in 200 μl of final volume (1 unit is the enzyme activity that completely cleaves 1 μg of DNA in 1 h at 37°C). Four restriction enzymes, *Eco*RV, *Dra*I, *Hpa*I and *Stu*I creating blunt-ended fragments, were used independently. The adaptor was ligated to genomic DNA overnight at 16°C in the presence of 10 U of T4 DNA ligase (Promega) in a final reaction volume of 20 μl, using the Universal GenomeWalker kit as indicated by Clontech. For PCR reactions the Advantage Genomic Polymerase mix from Clontech was used. GS1b primers were:

– AR1 (27mer, 48% GC content): 5′-GCAGGATTTGATCAACTTGGACCTCTC-3′;
– AR2 (26mer, 62% GC content): 5′-TCGGAGGCTCTGGCATGGATATCCGC-3′.The cycling parameters used were 94°C for 1 min and then 30 cycles of 94°C for 30 s, 68°C for 6 min with a final 68°C extension for 5 min. PCR fragments were subcloned into the plasmid vector pGEM-T easy (Promega) for detailed restriction mapping and DNA sequencing using the Sanger method (Sanger et al. 1977; the nucleotide sequence will appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number AF345985).

Transcription start site

The transcription start point was determined by primer extension analysis essentially as described by Sambrook et al. (1989) using the following primer: 5′-GTTGATCAAATCCGTC-3′. This sequence matched the nucleotides located from 9 to 24 downstream from the ATG initiation codon of the *GS1b* gene. The primer was allowed to hybridize for 1 h at 65°C. Total RNA (10 μg) extracted from embedded embryos, where the GS1b isoform is the main GS isoform present, was used as a template for the experiment.

RNA extraction and northern blot analysis

Total RNA was isolated from germinating seeds (3 days after imbibition, DAI) of *Pinus sylvestris*, and from seedlings with 2.5-cm-long cotyledons, 2, 4, 8 or 24 h after spraying with 50 μM gibberellic acid (GA_3). Control seeds/seedlings were untreated. Other hormones included in this study were: 50 μM abscisic acid (ABA), 50 μM indole-3-acetic acid (IAA), 5 μM N^6-benzylamin-

opurine (BAP), 5 μM zeatin and 1 μM thiadiazuron (TDZ). All hormones were supplied in the same manner as GA_3 and samples were harvested at the same time points. For northern analysis, 10 μg of total RNA was loaded per lane. The hybridization conditions using GS1a and GS1b probes were the same as described elsewhere (Avila et al. 2001a). Integrity and equal loading of RNA samples were verified by methylene blue staining (Wilkinson et al. 1990). Signal intensity was quantified using the Bioimaging analyzer BAS-1500 (Fujifilm España SA, Barcelona, Spain).

Database-assisted promoter analysis

The presence of putative *cis*-regulatory sequences was examined by bioinformatic approaches using three databases that identified transcription factor binding sites or *cis*-acting sequences in plant promoters:

i. PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/; Higo et al. 1999)
ii. Plant CARE (http://sphinx.rug.ac.be: 8080/PlantCARE/index.html; Rombauts et al. 1999)
iii. TRANSFAC (http://www.gene-regulation.de/; Wingender et al. 2001).

β-Glucuronidase (GUS) reporter gene constructions and expression in pine protoplasts

A 1,310-bp genomic fragment, containing 1,168 bp upstream and 142 bp downstream of the *GS1b* transcription start site, was used to construct a series of 5′-deletions of the GS1b promoter fused to the GUS reporter gene. This fragment was amplified between positions −1 and −1168 by PCR using appropriate primers. Three 5′-deletion fragments extending from positions −1 to −1022, −741 or −403 respectively, were also produced in the same way using the *Pfu* proof-reading Taq polymerase and the following primers:

- Forward, Pro1: 5′-ACTATAGGGCACGCGTG-3′ (−1168), Pro6: 5′-CACTCCACAAAACCCTG-3′ (−1019), Pro4: 5′-ACTCTTTCCCACGTTTT-3′ (−400) Pro5: 5′-AATGCA-TACGGAATCGT-3′ (−738).
- Reverse, Pro3: 5′-TCTGCTATTTCGAAATA-3′ (from −1).

The PCR products were ligated in-frame to the GUS gene in the pBI221 vector (Jefferson et al. 1987), replacing the CaMV 35S promoter. Four constructs were generated: GS1b-P1 (1,168 bp), GS1b-P2 (1,019 bp), GS1b-P3 (738 bp) and GS1b-P4 (400 bp). Two controls were also used in the transient-expression analysis: a positive control containing the CaMV 35S promoter, and a negative control, which was promoter-less, produced by deletion of the CaMV 35S promoter.

Protoplasts from maritime pine (*P. pinaster*) were isolated and transformed by electroporation as described previously (Gómez-Maldonado et al. 2001). Fluorometric assessment of GUS expression in pine protoplasts was accomplished according to standard procedures (Jefferson 1987) with minor modifications. Protein concentration was determined by the Bradford procedure (1976). The fluorometric assay reactions were carried out at 37°C and the fluorescence quantification was measured at 15-min intervals using an FL 6000 fluorescence reader (Bio-TEK) with methylumbelliferone (MU) as a standard. Histochemical detection of GUS activity with X-Gluc as substrate was carried out as described by Jefferson el al. (1987).

DNA-binding reaction and gel shift-assays

The sequences containing the putative AT and GA boxes were used for binding assays. Doubled-stranded oligonucleotides, sequences −791 to −767 and −768 to −747, containing the putative AT and GA boxes respectively were labeled by filling in with α-[^{32}P]dCTP and Klenow DNA polymerase. The radioactive probes were purified on polyacrylamide gels (Maniatis et al. 1982). Extracts of nuclear proteins were prepared as described by Willmitzer and Wagner (1981). Binding was carried out in 15 μl of 10 mM Tris (pH 8), 1 mM EDTA, 100 mM NaCl, 2 mM DTT, 10% glycerol and 2 μg of denatured salmon sperm DNA (binding buffer). The labelled DNA to be bound (1–2 ng) was incubated with 5 μg of crude nuclear extract from cotyledons or 10 μg from stems as the source of protein. Mixes were incubated for 30 min on ice. In unspecific-competition experiments, 0–0.5 μg of poly dI-dC was also included in the mixes, or 0–4 μg of poly dA-dT was assayed in standard conditions. In specific-competition experiments, the unlabelled DNA (0.1–1 μg) was included and assayed in standard conditions. In the proteinase K assay, the proteins were treated before the binding reaction with 3 μg of proteinase K for 10 min, at 25°C. At the end of the incubation period 1/10 of the mix volume of loading buffer was added, and then the samples were loaded on a 5% polyacrylamide 2% glycerol pre-electrophoresed gel. Running buffer was 0.5×TBE. Gels were electrophoresed in a coldroom at 10 V cm^{-1} for 2–5 h.

Protein determination

Proteins were quantified by the Bradford procedure using bovine serum albumin as standard (Bradford 1976).

Results

Cloning and structural analysis of the GS1b promoter

Using the PCR walking strategy we isolated a fragment containing approximately 1,300 bp of sequence upstream of the translation start site of the *GS1b* gene. The transcription start site was determined by primer extension using as a template total RNA extracted from imbibed embryos, where the *GS1b* gene product is the main product (Fig. 1). The transcription start site was determined to lie 68 bp upstream of the translation initiation codon and corresponded to a purine (adenine). This finding is in agreement with the position determined for most other *GS1* genes, where the transcriptional start site was found to be located between 46 and 90 bp upstream of the ATG (Forde et al. 1990; Marsolier et al. 1995) but differed from the position that was determined for the *GS1a* gene where the transcription start site was mapped to a site 180 bp upstream of the ATG.

Bioinformatic analysis was undertaken to identify conserved motifs found in other eukaryotic promoters and to find putative *cis* elements that could be operative in the regulation of *GS1b* gene expression (Fig. 2). A typical TATA box and a CAAT box-like sequence were identified at positions −38 and −124, respectively, relative to the transcription start site. The promoter sequence contained several putative regulatory elements including an AT box (−791 to −767; Huang et al. 1990) a putative gibberellin (GA)-response element (−768 to −747; Gubler and Jacobsen 1992) as well as three AC elements similar to *cis*-acting elements conserved in the promoters of genes encoding enzymes involved phenyl-

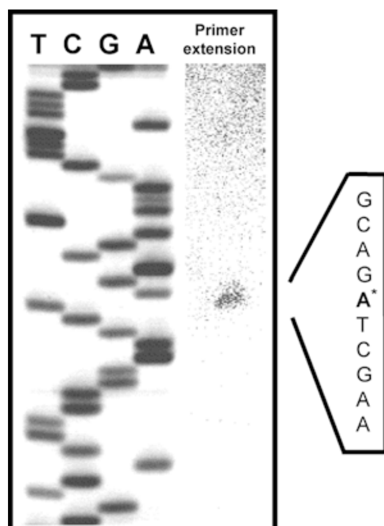

Fig. 1 Identification of the *GS1b* transcription start site by primer extension. Total RNA extracted from imbibed embryos of Scots pine (*Pinus sylvestris*) was used (10 μg) as well as the following primer 5′-GTTGATCAAATCCGTC-3′, which hybridizes to the N-terminal coding region of the gene. The sequence of the non-coding strand is shown in the figure (lanes TGCA) and is indicated next to the gel. The transcription starting point is indicated (*A**)

propanoid metabolism, such as phenylalanine ammonia-lyase (PAL; Gray-Mitsumune et al. 1999) (Fig. 2a,b).

Comparison of promoter organization between *GS1a* and *GS1b* promoters (Fig. 3a) showed that, while they both possessed obvious TATA boxes, CAAT boxes and transcription starting sites, they possessed no obvious shared sequence-specific motifs upstream of the transcriptional start site. In contrast, the sequence immediately downstream of the transcription start site is well conserved in both *GS* genes (Fig. 3b).

Expression of GS1b–GUS fusions in pine protoplasts

Transcriptional fusions of the *GS1b* promoter to the *uidA* (GUS) reporter gene were generated using the pBI221 vector, and designated as GS1b-P1, GS1b-P2, GS1b-P3 and GS1b-P4 (Fig. 4a). Following transformation of pine protoplasts by electroporation, transformants were examined for GUS expression. Since the gene expression is associated with vascular elements in shoots and leaves of pine seedlings, protoplasts from both sources were isolated and used for transient-expression experiments. The GUS expression pattern in protoplasts isolated from cotyledons differed from the pattern obtained when stem protoplasts were used (Fig. 4b). In protoplasts from cotyledons, the deletion of the first 149 bp of the promoter region increased the expression of the reporter gene almost 2-fold relative to the full-length promoter construct. This difference could be due to certain *cis*-acting elements located in that region that could have a negative regulatory role of the expression in the tissue, or to the presence of specific *trans*-activating factors expressed differentially in leaves and stems, perhaps reflecting a tissue-dependent control of the gene expression.

GA control of GS1b expression

Since the *GS1b* gene has a distinctive pattern of spatial and temporal regulation (Avila et al. 2001a), we determined if *GS1b* expression might be under hormonal control. The levels of *GS1b* transcripts were examined in developing pine seedlings treated with GA_3, ABA, BAP, TDZ, zeatin and IAA (Fig. 5). Northern blot analysis of transcripts derived from germinating seedlings (3 DAI) revealed that GA_3 treatment of seedlings induced a 2.5-fold increase in *GS1b* transcript abundance after 2 h treatment relative to control plants. None of the other hormones had an effect on the level of the *GS1b* transcript after 24 h of treatment, so we have concluded that they have no effect on the GS transcript level, at least under the experimental conditions tested. The observed induction of *GS1b* transcript accumulation with GA_3 appeared transient: 24 h after the treatment, the levels of the transcript started to decline. When cotyledons of pine seedlings (15 DAI) were used in the experiments (Fig. 5b) a similar induction effect was detected in the *GS1b* transcript level in green tissues.

Transcriptional regulation of GS1b by GA

Since *in silico* analysis of the GS1b promoter identified a putative GA-response element between −768 and −747 bp upstream of the initiation start site, we determined if there was any correlation between the presence of this element and the fact that *GS1b* transcript abundance increased in response to GA treatment. Transient-expression analysis of promoter–GUS fusions using pine protoplasts was used to determine if GA treatment could also alter reporter-gene expression. The cotyledon protoplasts treated with GA_3 had a similar level of reporter-gene expression relative to that observed for the four constructs in untreated protoplasts (Fig. 6, cotyledons) These results indicate that GA_3 did not induce an appreciable enhancement of the *GS1b* promoter activity in cotyledon cells, at least under the experimental conditions examined. In contrast, when stem protoplasts were used, increased GUS activity relative to controls was observed for protoplasts treated with GA_3 (Fig. 6, stems). This increase was observed when constructs GS1b-P1 and GS1b-P2 were used. The differences in expression of the reporter gene in cotyledons and stem protoplasts treated with GA could be explained by taking into account the fact that a higher proportion of vascular cells should be present in the stem protoplast preparation and this would facilitate the detection of *GS1b* expression. These findings indicate that *GS1b* promoter activity is responsive to GA_3 in

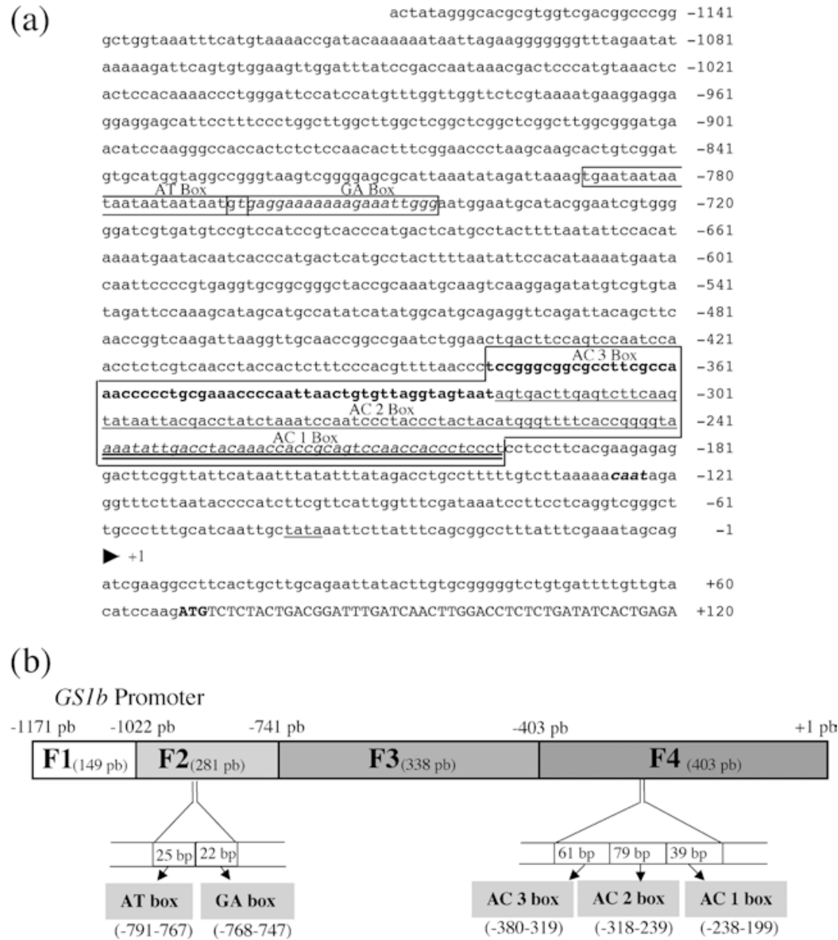

Fig. 2a,b Structural analysis of the Scots pine *GS1b* promoter. **a** Sequence of the 5′-upstream region of the *GS1b* gene. Nucleotides are numbered relative to the transcription start site (position +1). The putative *cis* elements are indicated (*boxes*). Since the AT and GA-response elements overlap by 2 bp, the box containing letters in normal font corresponds to the AT element, and that containing italic letters to the GA-response element. Putative AC elements are in a unique box where the AC3 element is in *bold*, the AC2 element is *underlined*, and the AC1 element is in *italics*. **b** Schematic of the *GS1b* gene promoter showing the location of the putative boxes involved in transcriptional regulation. The position of the transcriptional start site is indicated as +1. The numbers indicate the relative distance to the −1 nucleotide. F1, F2, F3 and F4 are the fragments deleted in the fusions to the GUS gene for expression analysis. The size of each fragment is indicated in bp. The putative *cis* elements detected in the database searching are specifically represented (size of each box as well as position relative to the −1). Boxes are quoted as *AT box* (AT-rich element), *GA box* (gibberellin-responsive element) and *AC boxes 1*, *2* and *3* (AC-rich elements, also present in genes expressed in xylem tissue)

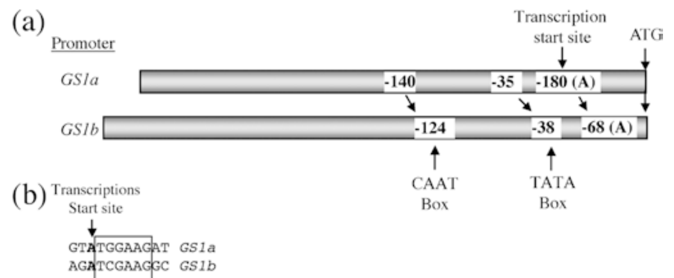

Fig. 3a,b Comparative diagram of Scots pine *GS* promoters: *GS1a* and *GS1b*. **a** Positions of putative CAAT and TATA elements are indicated. The transcription start site of both promoters is placed relative to the translation start codon (ATG). **b** Transcription start site is indicated in *bold* in both promoters. The conserved sequence of the downstream region is *boxed*

pine stem protoplasts, and suggests that the putative GA-response element identified may be operative in the control of *GS1b* expression in these cells. However, we cannot rule out the fact that an AT-box, which almost overlaps the GA-response element, or additional uncharacterized motifs in this region, might also be involved in the GA response. Interestingly, the GA response was abolished to a great extent (about 1/3 of the promoter activity) after the deletion of the GA/AT element in the construct GS1b-P3, reaching an even lower level than that observed in the untreated stem protoplasts.

Analysis of DNA–protein interaction in the 5′-region of the GS1b promoter

In order to further characterize the role for putative *cis*-acting elements in the regulation of the GS1b promoter, the interaction of pine nuclear proteins with the

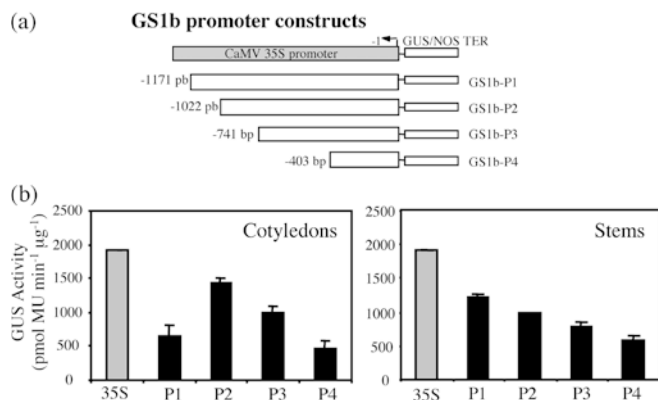

Fig. 4a,b Functional deletion analysis of the *GS1b* promoter from *P. sylvestris*. **a** Schematic of chimeric promoter–GUS constructs. Numbers indicate the distance (in bp number) relative to the *GS1b* transcription start site. The *gray box* indicates the *CaMV* 35S promoter region upstream of the transcription start point fused to the GUS reporter gene and used as positive control. *White boxes* represent the successive deletions of the *GS1b* promoter fused to the GUS reporter gene and designated as constructs GS1b-P1, which contains the full-length promoter, GS1b-P2, GS1b-P3 and GS1b-P4. **b** Expression levels of the constructs shown in **a**. GUS activity was determined as described in Materials and methods using *Pinus pinaster* protoplasts. Each value represents the average of three independent protoplast electroporation events with subsequent GUS quantification in triplicate. Error bars indicate SE values ANOVA ($P < 0.005$). GUS activity is represented by the *black rectangles* and the *grey rectangle* represents GUS activity of the positive control. GUS activity is expressed in pmol methylumbelliferone (MU) per min per μg protein

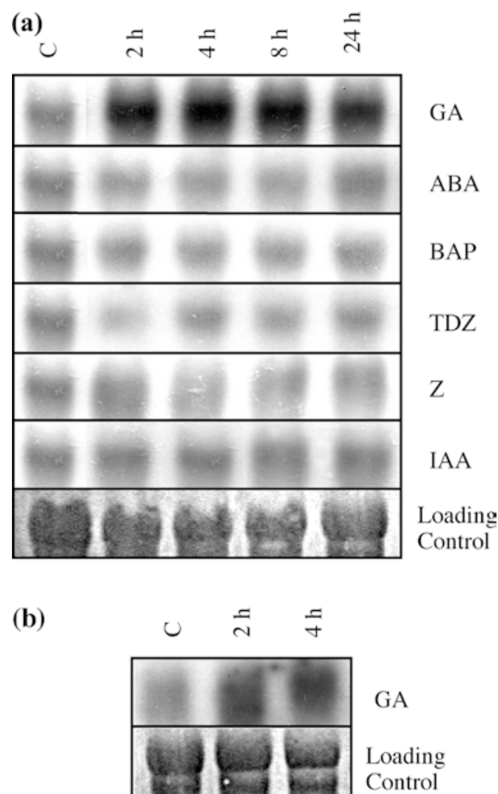

Fig. 5a,b Analysis of *GS1b* gene expression in gibberellin-treated Scots pine seeds and seedlings. **a** Seeds at 3 days after imbibition (3 DAI) were sprayed with 50 μM GA_3, 50 μM ABA, 50 μM IAA, 5 μM BAP, 5 μM zeatin (*Z*) or 1 μM TDZ, and GS gene expression was determined 2, 4, 8 and 24 h after treatment. Control (*C*) represents untreated plants. **b** RNA from seedlings with 2.5-cm-long cotyledons was sampled 2 and 4 h after GA_3 treatment. The loading control was the same for **a** and **b**, and was stained with methylene blue as indicated in Materials and methods

sequences between −791 and −747 was examined. Since the predicted GA-response element is very close to an AT element within the −768 to −747 region of the promoter, we determined if both sequences together (data not shown), or either one of them on its own, or neither of them, could bind nuclear proteins, as determined by electrophoretic-mobility-shift assays (EMSAs). As the GA-responsiveness of the *GS1b* promoter was contingent on the source of pine protoplasts, nuclear proteins from cotyledons were compared with those derived from stems to determine if any nuclear proteins could bind to the promoter region from −791 to −767 or from −768 to −747, respectively. In EMSAs, the migration of both putative *cis* elements was retarded by pine nuclear proteins (Figs. 7, 8). The AT element (Fig. 7) gave a stronger retardation complex with nuclear extracts from cotyledons than with nuclear proteins from stems. The binding appeared specific, as complex formation could not be inhibited by the non-specific competitor poly dI-dC, and complex formation was only reduced when 0.5 μg of competitor was included. On the other hand, the complexes with the AT element could be efficiently competed by poly dA-dT added to the mixes, and the complex disappeared completely with 0.5 μg of poly dA-dT. The formation of the retarded complex was protein-dependent as the treatment of nuclear extracts with proteinase K prior to the binding assay prevented the formation of the retarded complex (Fig. 7).

In contrast to the results obtained with the AT element, when the EMSAs were conducted with the predicted GA-response element the retarded complex was dependent on the source of the nuclear proteins (Fig. 8). Only nuclear proteins from stems were able to form complexes with the GA-response element. No DNA–protein complex formation was observed when nuclear proteins extracted from cotyledons were used. The absence of a retarded complex in the shift assays with nuclear proteins from cotyledons could be explained by considering that the *trans*-acting factor represents a small fraction of the total nuclear protein extract in this particular tissue where many other proteins involved in cellular processes (e.g. photosynthesis) should be present. If this is the case, the retarded complex would hardly be visible. The complex could not be competed for by the addition of the non-specific competitor poly dI-dC, or by poly dA-dT. The stem nuclear protein had a high affinity for the putative GA-response element and complex formation was only efficiently competed for when 1 μg of cold specific competitor was present. As was the case with the AT element, complex formation

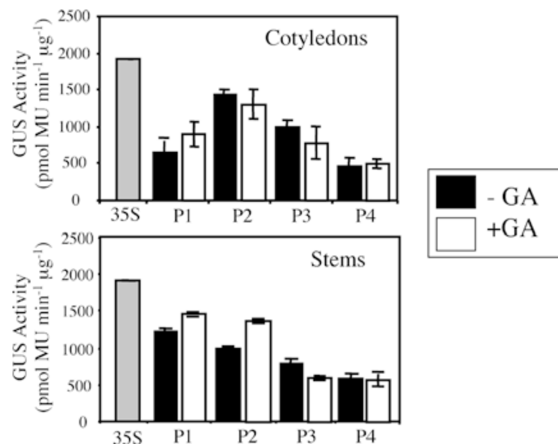

Fig. 6 Effect of GA treatment on *GS1b* promoter activity. Expression levels of the constructs shown in Fig. 4 were analyzed simultaneously in electroporated protoplasts from *P. pinaster* that were incubated in buffer containing (*white columns*) or not containing (*black columns*) 1 µM GA_3. Each value is the average of three independent protoplast electroporation events and three GUS quantification assays. ANOVA ($P=0.005$)

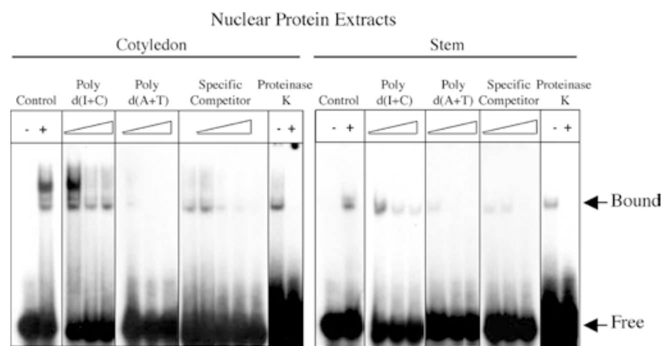

Fig. 7 Electrophoretic Mobility Shift Assays (EMSAs) with the AT-box (−791 to −767) oligonucleotide. EMSAs were performed with 5 µg of nuclear protein extract from Scots pine cotyledons and 10 µg from stems, using 2 ng of the labelled AT probe (−791 to −767) derived from the *GS1b* gene. The negative control corresponds to the binding mix without protein. The positive control is the standard binding mix containing 2 ng of labelled fragment, 5 µg of protein (or 10 µg, as appropriate) and 2 µg of herring sperm DNA. To the binding mixes prepared as before, poly dI-dC was added at the following concentrations: 0.1, 0.3 and 0.5 µg in each binding mix. The poly dA-dT competitor was added at 0.5, 2 and 4 µg. Cold oligonucleotide as specific competitor was added at the following concentrations: 100, 300, 500, 900 and 1,000 ng in the cotyledon nuclear protein experiments; in the stem protein experiments, only the three lower concentrations are shown. The proteinase K experiments were conducted as described in Materials and methods

with the predicted GA-response element was also protein-dependent.

Discussion

In a number of woody perennial species, organic nitrogen is stored in specialized tissues such as bark, to be further remobilized for the formation of developing organs in periods of active growth (Coleman et al. 1994). In woody perennial plants, the reaction catalyzed by cytosolic glutamine synthetase (GS1) may be a major step in controlling plant growth and development because it combines the re-assimilation with transport of nitrogen released in metabolic processes (Suárez et al. 2002). In pine, two GS1 genes have been cloned and their expression patterns studied: *GS1a* (Cantón et al. 1993, 1999) and *GS1b* (Avila et al. 2000, 2001a). These genes encode distinct GS1 polypeptides, which are assembled into homo-octameric cytosolic isoforms of the GS holoenzyme, GS1a and GS1b, with different molecular and kinetic properties (Avila et al.1998; de la Torre et al. 2002). Studies on the regulation of *GS1* gene expression have also been initiated by the functional characterization of the *GS1a* promoter (Avila et al. 2001b). In this paper the molecular cloning and functional characterization of the 5′-upstream region of the pine (*Pinus sylvestris*) *GS1b* gene is presented for the first time. The analysis of the regulatory region of the *GS1b* gene reported here provides new insights that highlight the physiological roles that may be played by this particular *GS1* gene in conifers.

A comparison of the structures of *GS1a* (Avila et al. 2001b) and *GS1b* (Fig. 3a) promoters reveals that the regulatory regions of both pine *GS1* genes are largely unrelated, with only a short stretch of conserved sequence (Fig. 3b), which includes the transcription start nucleotide and six nucleotides downstream of this position. The conservation of the transcriptional start site may be indicative of a sequence requirement for the RNA polymerase binding to establish the basal transcriptional complex. Since the number of characterized conifer promoters is still limited we cannot conclude whether this conserved sequence is specific for GS or if it is present in other conifer genes. The structural differences in the regulatory regions of pine *GS1* genes supports the finding that these genes have distinct expression patterns during plant development (Avila et al. 2001a; Suárez et al. 2002), and suggests that the

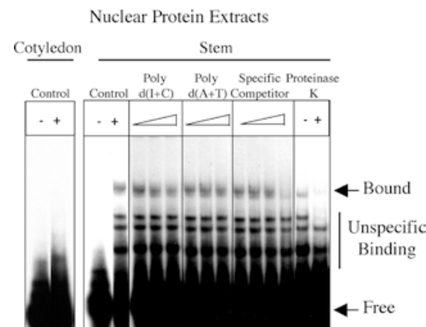

Fig. 8 EMSAs with the DNA derived form the putative GA-responsive element (−768 to −747) from Scots pine. The concentrations of added protein and unspecific DNA were the same as described for the AT-box experiments. Concentrations of cold fragment used in the specific competition experiments were: 250, 500, 750 and 1,000 ng per mix. Proteinase K experiments were performed as described in Materials and methods

distinctive patterns of regulation may have evolved long ago.

Gene expression of pine *GS1b* is mainly associated with the vascular bundles in all tissues of the pine seedling, including cotyledons, the hypocotyl and roots (Avila et al. 2001a). This pattern is similar to that found in the embryo, suggesting that this gene plays an important role in N transport or translocation within the plantlet, even at very early stages of the development. These findings suggest that the expression of *GS1b* is tightly regulated by a number of factors that vary in concentration and/or activity during pine seedling development, as well as specific signals controlling expression in particular cell types.

As a step toward understanding the regulatory mechanisms controlling *GS1b* gene expression, we have begun to delineate the functional elements present in the *GS1b* promoter. *In silico* analysis of the promoter revealed the presence of several putative *cis*-acting elements in the *GS1b* promoter. Notably, a subset of the predicted functional motifs has previously been implicated in GA-regulated gene expression. It has been shown that three to four cis-acting promoter elements are necessary for GA induction of α-amylases (Gubler and Jacobsen 1992). Similarly, we have identified several putative GA-response motifs with the region between −1022 and −741 bp of the *GS1b* promoter. In order to assess the importance of these motifs in the regulation of *GS1b* expression, a functional dissection of the promoter sequences was initiated.

Deletion analysis of the *GS1b* promoter showed a progressive decrease in reporter gene activity when the regions of promoter distal to the transcriptional start site were removed (Fig. 4). These data confirm the presence of functional *cis*-acting elements at different positions in the structure of the *GS1b* promoter and suggest that these elements may function as targets for a variety of *trans*-acting factors. Interestingly, in contrast to most of the deletions, deletion of the 5′ most distal part of the *GS1b* promoter resulted in increased reporter activity, exclusively in cotyledons. This finding suggests that sequences lying in this region may normally function to repress transcription in the cotyledons. Further studies are needed to determine the extent to which these sequences are involved in the tissue- and cell-specific regulation of gene expression.

Among the various GS gene promoters studied to date, the *GS1b* promoter is the sole example where the expression of the gene is controlled by GA. It is well known that GAs constitute one of the major classes of phytohormones and that they are involved in many aspects of plant growth and development, including elongation growth, dormancy release and seed germination (Bewley 1997; Yaxley et al. 2001). In this context, we have previously reported that the *GS1b* gene has an important role in the regulation of the nitrogen flux in germinating seeds. In *Pinus sylvestris*, protein hydrolysis begins in the embryo (Simola 1974). Detailed analysis of gene expression indicated that *GS1b* was expressed in the early stages of germination, during the re-assimilation of ammonium released in the breakdown of embryo storage proteins (Avila et al. 2001a). Amino acid biosynthesis at this time may be critical for the de novo protein synthesis related to the loss of seed dormancy (Schneider and Gifford 1994). Both *GS1b* mRNA and GS1b polypeptide are abundant in the pine embryo (Avila et al. 2001a), particularly in the central area of the embryo. This is the area where protein vacuoles are more rapidly mobilized (Stone and Gifford 1997), probably due to the high demand for amino acids that takes place during radicle expansion and differentiation (root protrusion).

Exogenous GA was able to induce *GS1b* expression in germinating seeds (Fig. 5), suggesting the existence of hormonal control of nitrogen re-assimilation and translocation in the early stages of pine development. Hormonal regulation of ammonium assimilation in trees has been recently reported, e.g. in the latex of rubber tree, transcripts for the cytosolic GS accumulate in response to ethylene (Pujade-Renaud et al. 1997). However, the work described herein is, to the best of our knowledge, the first report of regulation of GS1 gene expression by GA. Interestingly, the observed increase in *GS1b* expression in response to GA was apparent by 2 h after the start of the addition of GA, suggesting rapid transcriptional activation of *GS1b* gene expression. In contrast, ethylene induction of *GS* expression in the rubber tree required a longer period (6–12 h). Clearly, the *trans*-acting factors that interact with the GA-responsive region of the *GS1b* promoter must do so relatively rapidly, at least in comparison to the ethylene response in the rubber tree. The pine stem nuclear proteins that bind to the predicted GA-response element in the *GS1b* promoter with high affinity (Fig. 8) are good candidates for the *trans*-acting factors involved in the GA regulation of the *GS1b* gene. Future studies focused on the characterization of these proteins should begin to uncover the molecular mechanisms underpinning the regulation of this gene by GAs.

In pine seedlings, the abundance of *GS1b* transcripts in the hypocotyl and roots, and the precise localization of gene expression in the vascular bundles support a role for the *GS1b* gene product in some aspect of vascular cell functions. The precise role of *GS1b* in vascular development remains to be determined, but it is tempting to speculate that it may play an important role in N recycling in xylem cells. The *GS1b* product may function in the developing xylem cells, where PAL activity is high, to re-assimilate the liberated ammonium. In keeping with this hypothesis, the proximal region of the *GS1b* promoter contains AC elements, which are frequently found in the promoters of genes encoding enzymes involved in lignin biosynthesis, particularly PAL (Gray-Mitsumune et al. 1999). Currently we are examining the functional significance, if any, of such putative regulatory elements.

In conclusion, the results of this study extend our knowledge of the regulation of *GS1b* expression by the identification of *cis* elements in the promoter region of

the gene. Further work is needed to characterize the nature of the nuclear factors involved in the transcriptional control of *GS1b* gene expression.

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