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doi: 10.1111/j.1399-3054.2004.00353.x
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Interaction of *cis*-acting elements in the expression of a gene encoding cytosolic glutamine synthetase in pine seedlings

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Received 3 March 2004

Glutamine synthetase (GS) genes, *GS1a* and *GS1b,* in pine (*Pinus sylvestris* L.) are differentially regulated in tissue specificity and during seedling development. To gain insight into the regulatory mechanisms controlling their expression, we have analysed the 5′-flanking sequences of the gene *GS1a* using a transient expression system in pine protoplasts. Structural analysis of this region revealed the presence of putative regulatory elements including two AT-rich elements and a poly CT consensus sequence. A series of 5′- and 3′-deletions of the untranslated region covering the three putative elements, −800 to −626, −626 to −427 and +118 to +177 were analysed to demonstrate the functional implications of these elements in gene regulation. An electrophoretic mobility-shift assay showed that nuclear proteins prepared from pine cotyledons interact with both AT-rich regions (−800 to −427). Interestingly, no protein binding was detected when the untranslated region (+118 to +177) was included, even if deletion of that region suppressed promoter activity in the transient expression experiments conducted. However, simultaneous deletion of both types of *cis* elements, A/T and CT, resulted in a recovery of promoter activity of 50%. These results suggest a key regulatory role of the CT box by the interaction with A/T stretches in the distal part of the promoter and possibly with the proximal region (−427 to −1).

Introduction

Glutamine Synthetase (GS) is the enzyme responsible for the primary assimilation of ammonium produced by nitrate reduction or fixation of dinitrogen as well as the reassimilation of ammonium released by photorespiration and other metabolic processes. The various roles of GS in plant metabolism are undertaken by different isoforms encoded by a small multigene family (Forde et al. 1989), whose members are expressed developmentally and in response to external stimuli. These GS isoforms are located in different compartments of cells (cytosol or chloroplast) and different tissues and organs of plants (Hirel et al. 1993).

As occurs in angiosperms it seems that a small multigene family could be operative in gymnosperms (García-Gutiérrez et al. 1998, Ávila et al. 2001a). Two distinct but homologous nuclear genes for GS have been localized closely linked in the pine genome (Ávila et al. 2000). These isogenes encode cytosolic isoforms of the GS enzyme in conifers, GS1a and GS1b with different molecular and kinetic properties, which are differently expressed in pine seedlings (Ávila et al. 1998, 2001a).

Molecular data from the characterization of a GS1a cDNA clone showed that the *GS1a* gene is actively expressed in chloroplast-containing tissues of the pine seedlings (Cantón et al. 1996) and the transcript levels were affected by development and the light regime (Cantón et al. 1999). To go further in this study, we previously described the isolation of a partial genomic clone containing 7 exons of the coding region of the *GS1a* gene (Ávila et al. 2001b) and the characterization of the 5′-untranslated region. This region was fused to the reporter gene *uidA* and transient expression analysis performed in pine cotyledons transformed by microprojectile bombardment. Stable expression analysis in transgenic *Arabidopsis* was also studied (Ávila et al. 2001b). The 5′-untranslated region of the gene was functional as a promoter in both homologous and heterologous systems and appeared to contain regulatory sequences involved in transcriptional activation by light

(Ávila et al. 2001b). Furthermore, very recently the function of other gene promoters from conifers has also been characterized in heterologous systems (No et al. 2000, Ciavatta et al. 2002, Forward et al. 2002). All these studies are contributing to a great extent to a better understanding of transcriptional regulation in this important group of trees. However, the available information on the molecular nature of the regulatory elements involved in gene regulation and how these elements interact to drive gene expression still remains obscure. Since *GS1a* gene is expressed in a light-dependent manner (Cantón et al. 1999) we wanted to verify the existence of putative *cis*-regulatory elements in the promoter region that may be involved in gene regulation. Sequence analysis revealed the presence of two AT-rich regions similar to those previously described in the *rbcS* and *cab* genes (Castresana et al. 1988, Datta and Cashmore 1989, Ueda et al. 1989) and the nuclear gene encoding chloroplast glutamine synthetase from pea (Tjaden and Coruzzi 1994). AT-rich repeated sequences have also been reported to be present in the proximal and distal regions of the cytosolic soybean GS promoter (Reisdorf-Cren et al. 2002). In addition a poly CT consensus sequence previously characterized in the *Arabidopsis HMG* 2 gene was also identified (Enjuto et al. 1995).

In this paper, the relevance of these putative elements in the regulation of GS1a expression has been examined by using two different approaches: transient expression in a homologous pine protoplast system and gel retardation analysis. We have determined that two AT-rich stretches placed in the most distal part of the promoter are essential for its activity, and formed retarded complexes in shift assays involving factors present in nuclear extracts from pine cotyledons. Experimental evidence is also present to support an interaction of AT and CT elements in transcriptional regulation of the glutamine synthetase gene.

Materials and methods

Construction of chimeric plasmids

A transcriptional fusion of the *GS1a* promoter (accession number AJ225121) to a β-glucuronidase (GUS) reporter gene was made as follows. The DNA sequence between −800 and +180 of the *GS1a* gene was PCR-amplified with Pl-1 (−800 to −783) and Pl-2 (+180 to +163) primers (Fig. 1). *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA), which has proofreading activity, was used to enhance the fidelity of PCR amplification. (*Pfu* DNA polymerase was used for all the following PCRs.) Next, the 980 bp fragment was cloned into the promoter-less GUS plasmid (pBI221) cut with *Sma*I (promoter-less GUS-NOS gene in PBI221). The integrity of its sequence was checked by sequencing (Sanger et al. 1977). To create a series of 5′ and 3′ deletions of the promoter primers Pl-3 (−626 to −609), Pl-4 (−427 to −410) and Pl-5 (−1 to −18) were used (Fig. 1). The amplified fragments

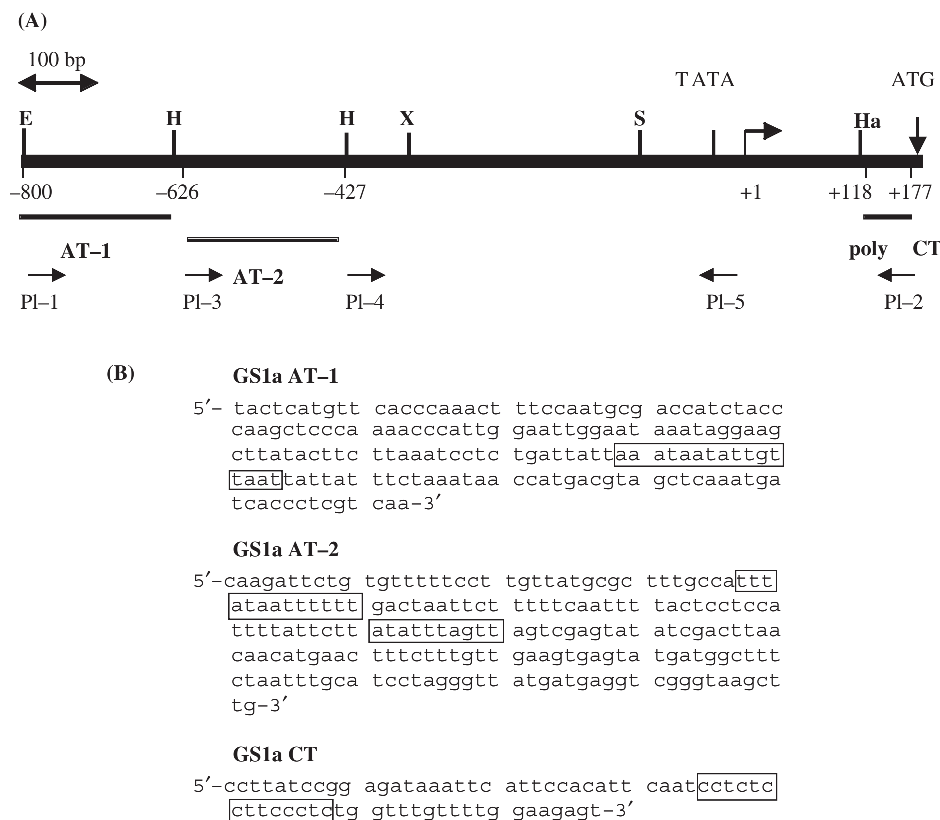

Fig. 1. (A) Schematic representation of the *GS1a* promoter showing the fragments used to study DNA–protein interactions. TATA box and translation start site are also indicated. Nucleotides are numbered relative to the transcription start site (position + 1). Primers used to generate GUS constructs are indicated by arrows. (B) Sequence of DNA fragments: AT-1, AT-2 and CT used in the study of DNA–protein interactions. Putative binding elements are indicated in square boxes.

were subcloned in the PBI221 promoter-less plasmid upstream the β-glucuronidase (GUS) reporter gene.

Plant material

Scots pine seeds (*Pinus sylvestris* L.) used in all experiments were from Servicio de Material Genético, Instituto de Conservación de la Naturaleza, Madrid, Spain. Seeds were imbibed in deionized water for 12 h under continuous aeration then they were germinated in a plastic pot containing vermiculite (Eurover, Europerlita, SA, Almería, Spain) as support. Seedlings were grown in a controlled culture chamber (Ibercex H-900-B, ASL, SA, Madrid, Spain) at 24°C using a 16 h light/8 h dark photoperiod. Illumination was provided by fluorescent lamps (Sylvania F-48T12/CW/WHO; Koxka, Pamplona, Spain) at a flux density of 150 μmol m^{-2} s^{-1}. Plants were watered regularly but no nitrogen fertilization was supplied. Samples were harvested at the same time in the light/dark cycle.

Protoplast isolation and electroporation

Cotyledons from seedlings 12- to 15-days-old were used as starting material. Splitting the cotyledon fragments longitudinally 2–4 times and then cutting transversely into 2- to 5-mm thick pieces facilitated enzyme penetration. Cells from cotyledons were released by incubation overnight in the dark at 25°C in an enzyme mixture containing 0.4% (w/v) cellulase (Calbiochem, Darmstadt, Germany), 0.4% (w/v) macerase (Calbiochem), 0.4% sucrose and 0.44% (w/v) K3 medium as described previously (Gómez-Maldonado et al. 2001). Protoplasts were resuspended to a 1×10^5 ml^{-1} final density in the electroporation medium and 1 ml was mixed with 50 µg supercoiled plasmid DNA (PBI 221 derivatives) and 10 µg of sheared salmon sperm DNA as carrier and electroporated in a pulser^{tm} cuvette (Bio-Rad, Barcelona, Spain) of 0.2 cm electrodes. A capacitor discharge system was used to deliver an 800 V cm^{-1}. After the electrical discharge, protoplasts were left at room temperature for 10 min and then diluted with a volume of 0.5 M mannitol, 88 m*M* sucrose and 0.5 mg l^{-1} carbenicillin. The protoplasts were cultured in the dark at 24°C for 36 h. The β-glucuronidase activity was determined using a fluorimetric assay (Jefferson et al. 1987). Before the enzyme assay, the protein concentration in the supernatant was adjusted to 1 mg ml^{-1} (Bradford 1976), 75 µl of extract was added to 500 µl grinding buffer (Jefferson et al. 1987) containing 1 m*M* 4-methylumbelliferyl glucuronide, prewarmed to 37°C. Reactions were carried out at 37°C and the fluorescence was measured at 15 min intervals using a FL 6000 fluorescence Reader Bio-TER (excitation 365 nm, emission 455 nm) as described (Jefferson et al. 1987).

DNA probes

The *GS1a* promoter region from −800 to −627 was PCR amplified with a forward (5′-GGACTTTACTCATGTT-CACCCAA) and a reverse primer (5′-TGTCTAAGCT-TTTGACGAGGGTGATCAT) which contain *Bam*H1 and *Hin*dIII restriction sites, respectively, at their 5′ ends (underlined). The PCR product was digested with *Bam*H1 and *Hin*dIII, and the resulting fragment was cloned into pBlueScript SK^- (Stratagene). The plasmid construct was verified by DNA sequencing. For electrophoretic mobility-shift assays, the DNA fragment was cut out from the plasmid, purified from agarose gels, and end labelled with [α-^{32}P]dCTP using the Klenow enzyme.

Shorter fragments containing the putative AT-1 (−720 to −661) and AT-2 (−503 to −461) elements were currently used for DNA-binding reactions. Complementary oligonucleotides were allowed to anneal, and the probes were ^{32}P-labelled with Klenow DNA polymerase, and purified on polyacrylamide gels.

Nuclear extracts

A crude preparation of pine cotyledon nuclei was obtained using a procedure modified from Willmitzer and Wagner (1981). Cotyledons of 2.5 cm in size were harvested into liquid nitrogen and stored at −80°C to be used as a source of nuclei. Plant material (10 g FW) was ground in liquid nitrogen in a mortar. All subsequent steps were carried out at 4°C. The powder was then homogenized in grinding buffer (7.5 ml g^{-1} of tissue). Grinding buffer consisted of 0.25 M sucrose, 10 m*M* NaCl, 10 m*M* MES pH 6.0, 1,10-phenantroline, 0.15 m*M* spermine, 0.5 m*M* spermidine, 20 m*M* β-mercaptoethanol, 0.2 m*M* phenylmethyl sulfonyl fluoride, 0.01% Triton X-100, the pH was adjusted to 5.3. After filtering thorough three layers of muslin the homogenate was centrifuged 15 min at 500 *g*. The pellet was then washed three times in washing buffer (the same as homogenization buffer minus Triton X-100) using 2.5 ml g^{-1} of tissue, spinning for 7 min at 500 × g each time. The procedure for extraction of nuclear proteins was modified from Siebenlist et al. 1984). The nuclear pellet was resuspended in 1 ml of a solution containing 10 m*M* Hepes pH 8, 300 m*M* NaCl, 25% (W/V) glycerol, 0.1 m*M* EGTA, 0.1 m*M* EDTA, 5 m*M* spermidine, 0.15 m*M* spermine, 1 m*M* phenylmethylsulphonyl fluoride, 7 m*M* β-mercaptoethanol and mixed for 2 h at 4°C. Debris was removed by centrifugation at 12000 *g* for 10 min. The crude nuclear extract obtained was divided in aliquots, frozen immediately in liquid nitrogen and stored at −80°C. Protein concentration in the extracts was determined by Bradford's procedure (1976).

Gel retardation analysis

Binding was carried out in 15 µl of 10 m*M* Tris (pH 8), 1 m*M* EDTA, 100 m*M* NaCl, 2 m*M* 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 4 µg of crude nuclear extract as a source of protein. Mixes were incubated for 30 min on ice. In unspecific competition experiments 0–1 µg of poly dI-dC or 0–1 µg

of poly dA-dT was also included in the mixes. In specific competition experiments, the unlabelled DNA (0.25–1 μg) was included and assayed in standard conditions. At the end of the incubation period 1/10th of the mix volume of loading buffer was added and samples were loaded on a 5% polyacrylamide 2% glycerol pre-electrophoresed gel. Running buffer was 0.5 × TBE (buffer Tris borate EDTA). Gels were run in the cold room at 10 Vcm^{-1} for 2–5 h.

Results and Discussion

Functional analysis of the 5′flanking region of *GS1a* gene from Scots pine

In a previous study it was shown that the 980 bp 5′-untranslated region of the *GS1a* gene is able to drive GUS reporter gene expression either in homologous and heterologous systems (Ávila et al. 2001b). The region seems to contain regulatory sequences involved in the transcriptional activation of the gene by light and the location of expression in pine seedlings was maintained in transgenic *Arabidopsis*. Figure 1A shows in a diagrammatic form the structure of the 5′untranslated region of the *GS1a* gene used in this study. Regions containing putative *cis* elements named AT-1, AT-2 are located in the most distal part of the promoter whereas the poly CT element is placed in the transcribed but untranslated region. The sequences of *GS1a* AT-1, AT-2 and CT regions are shown in Fig. 1B.

To determine whether or not these elements placed in the 5′flanking sequence of the *GS1a* have an essential role in the transcription of the gene, the 980-bp region upstream of the translation start site (−800 to +180) was fused to the GUS reporter gene in pBI221 (Jefferson 1987) resulting in the construct named pGS1 (Fig. 2A). Two different types of constructions were created to investigate the function of these upstream elements on the *GS1a* promoter. The first type of constructs, pGS2 (−626 to +180) and pGS3 (−427 to +180), were 5′ deletions that included the untranslated region of the gene and where AT-1 and AT-2 regions were successively deleted. The second type, pGS4 (−800 to −1), pGS5 (626 to −1) and pGS6 (−427 to −1), were constructs where the transcribed but untranslated (5′UTR) region was excluded and the A/T stretches present in the promoter were successively deleted (Fig. 2A). The different constructs were electroporated into protoplasts isolated from pine cotyledons and GUS activity was determined as described in Section Protoplast isolation and electroporation of Materials and methods. The results of transient expression assays using the chimerical constructs are shown in Fig. 2B. Deletion of AT-1 motif from −800 to −626 resulted in a 20% decrease of GUS activity. However, when both A/T rich stretches (from −800 to −427), were deleted no GUS expression was detected indicating that this region must be critical for expression of the *GS1a* gene. The importance of AT elements in the regulation of GS genes has been previously reported in angiosperms (Forde et al. 1990, Tjaden and Coruzzi 1994, Reisdorf-Cren et al. 2002). When the 5′UTR region was not included in the constructs no GUS activity was observed, even if the A/T stretches were present (constructs pGS4, pGS5). This finding is in agreement with our previous results (Ávila et al. 2001b), where a deletion of the poly CT element placed in the transcribed but untranslated region of *GS1a* gene decreases the expression of the reporter gene in germinating pine embryos and transgenic *Arabidopsis*. This indicates that the region placed between +1 and +180 upstream the initiation is important for *GS1a* gene expression. However, in pGS6 construct where the A/T rich segment was deleted and the 5′UTR was not included, the promoter activity was restored up to 50% relative to the pGS1 construct. This could indicate the presence of a negative element in the region placed between −626 and −427 that affects *GS1a* gene expression, and when deleted the promoter activity is restored. However, since the difference between pGS3 and PGS6 is the presence or not of the untranslated region of the gene containing the poly CT element, the observed effect can only be attributed to this difference. Our data also indicate differences in the relative importance of AT1 and AT2 in the regulation of the promoter. The simultaneous absence of AT-1 +AT-2 sharply decreases reporter gene expression, whereas only a partial decrease was observed when AT-1 was removed. Interestingly, the absence of AT-1 + AT-2 restored a substantial part of the reporter gene expression lost when the 5′UTR region was simultaneously removed, whereas the exclusive absence of AT-1 had no effect. These results highlight the importance of both AT fragments in the control of pine GS transcription.

Analysis of DNA–protein interactions in the 5′ flanking region of the *GS1a* gene

As an additional step in the characterization of *cis*-acting elements in the *GS1a* promoter, we have analysed the in vitro interactions between nuclear factors extracted from Scots pine cotyledons and sequences in the upstream region of the gene by electrophoretic mobility shift assays (EMSAs). Initially, the DNA fragments used in this study were the entire regions shown in Fig. 1. Both fragments AT-1 and AT-2 corresponded to the previously deleted regions analysed in transient expression experiments pGS2 and pGS3, and the poly CT element included in the deleted region in constructs: pGS4, pGS5 and pGS6. Figure 3 presents the results obtained when fragments AT-1, AT-2 and CT were tested in the shift assay. Both A/T rich fragments formed complexes that migrated more slowly than free DNA and that were not seen in the absence of nuclear proteins, but no retardation complex was visible with the CT fragment.

The AT-1 region contains an A/T rich element similar to *rbcS* genes previously described (Hutchinson et al. 1990, De Rocher et al. 1993, Hanania and Zilberstein 1994). Interaction of AT-2 region with nuclear extracts

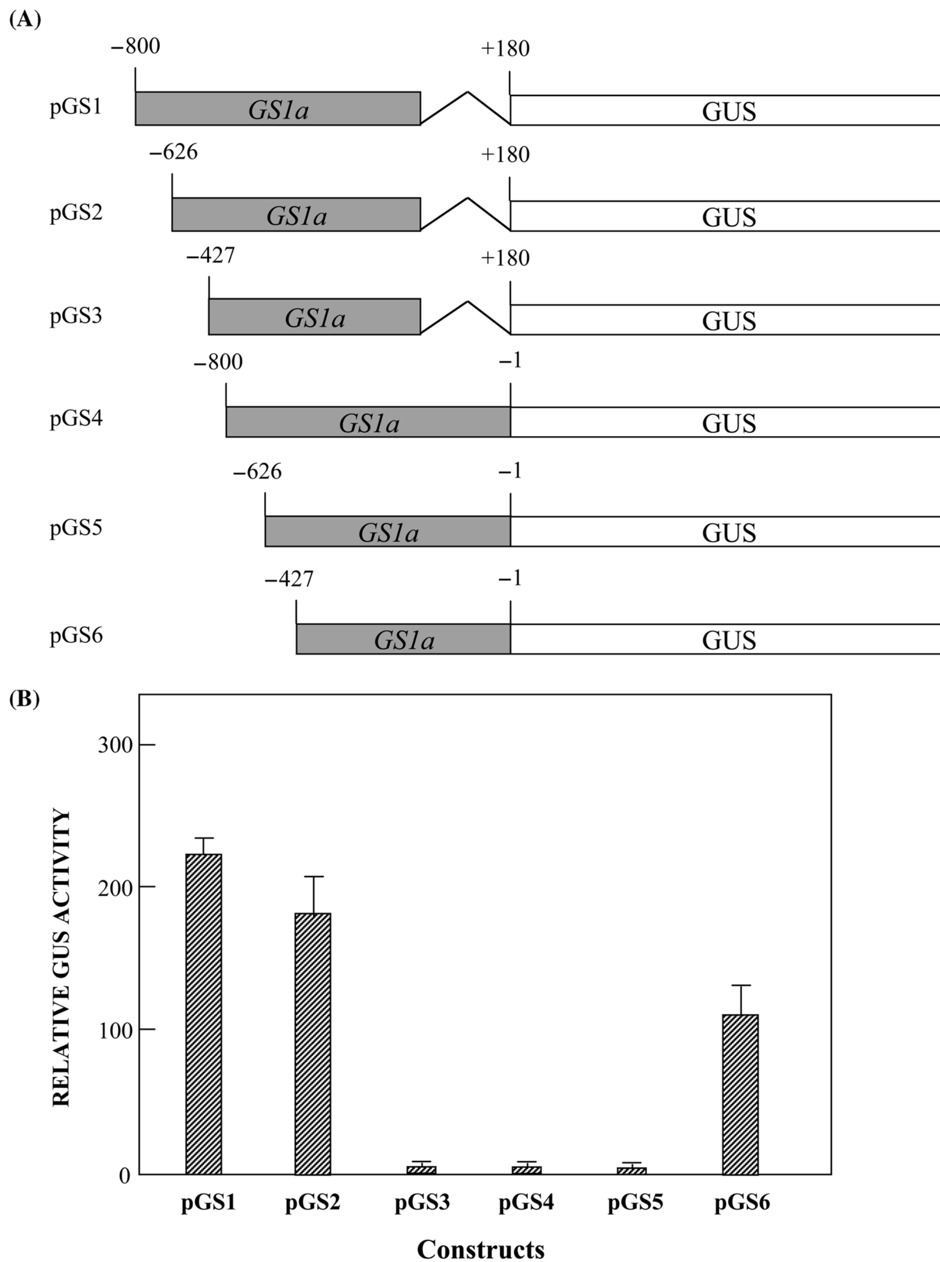

Fig. 2. (A) Schematic diagram of chimeric *GS1a* promoter-GUS contructs. Numbers indicate the distance relative to the *GS1a* transcription start site. Angled line represents the 5′-transcribed but untranslated (5′UTR) region. Translation initiation starts at position + 180. The light-grey boxes indicate the *GS1a* promoter region upstream the transcription start point. White boxes indicate GUS reporter gene sequence. (B) Expression levels of the construct shown in (A). GUS activity was calculated as described in section Protoplast isolation and electroporation of Materials and methods. Each value represents the average of three independent protoplasts electroporation events and GUS quantification. Error bars indicate SE values. A relative GUS activity value of 100 correspond to 10 pmol of 4-methylumbelliferone μg^{-1} protein min^{-1}.

could be explained on the basis of similarities with AT-rich stretches present in other GS promoters from angiosperms (Forde et al. 1990, Tjaden and Coruzzi 1994). No DNA–protein interactions were detected within the region of the proximal sequence in the promoter placed between −427 and −1 (data not shown). However, we cannot rule out that some retardation complexes could be seen in that region in experimental conditions different from the one used here. The poly CT element was included in this study to evaluate three considerations: (1) some poly CT elements with regulatory properties, placed in the untranslated region, had been previously reported in other genes (Enjuto et al. 1995), and made it a likely target site for transcriptional regulatory factors; (2) a deletion of the poly CT element decreased transient GUS expression in pine cotyledons (Avila et al. 2001b); and (3) when the 5′ untranslated region containing the poly CT element was omitted in the constructs (Fig. 2) no GUS activity was observed, indicating the presence of a sequence essential for the control of the promoter. However, in spite of these considerations no DNA–protein interaction was detected within the region of the promoter containing the poly CT element (Fig. 3).

Characterization of the binding complexes

Since the regions under study were rather large, we decided to further characterize AT-1 and AT-2

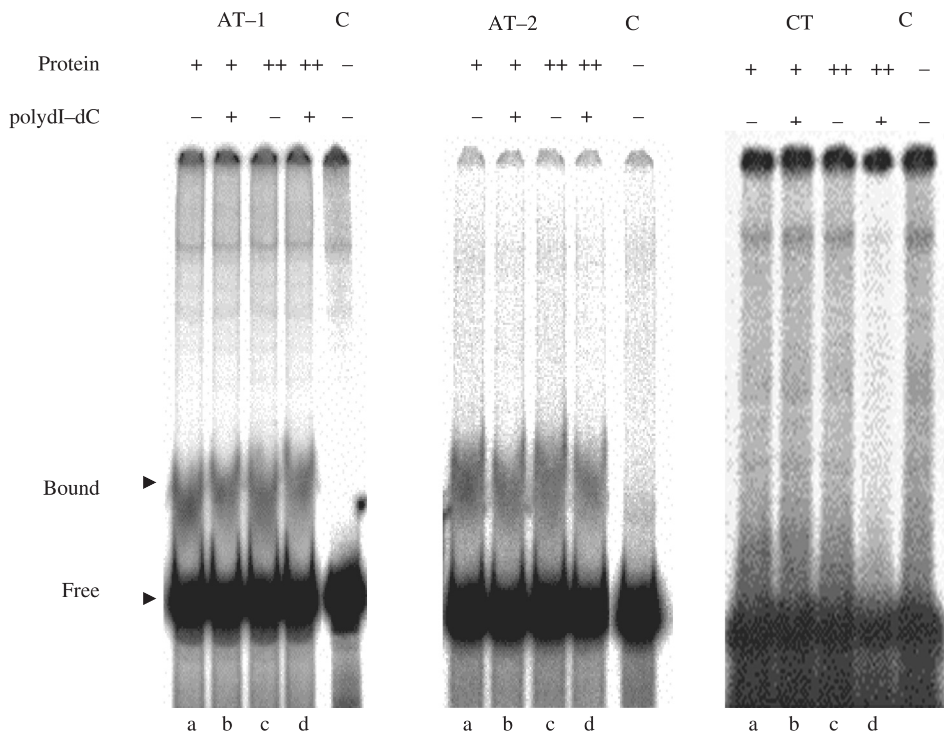

Fig. 3. Gel retardation assays performed with protein nuclear extracts from pine cotyledons and DNA fragments AT-1 and AT-2 and CT derived from *GS1a* gene. Lanes a, b with 4 µg of protein nuclear extract and 0 or 0.5 µg of poly dI-dC. Lanes c and days the same except 5 µg of protein nuclear extract was included. Lane C is a control without protein nuclear extract. All samples contained 2 µg of herring sperm DNA.

complexes by performing control experiments using as targets the boxes containing the core putative sequences, 60 bp for AT-1 (−720 to −661) and 43 bp for AT-2 (−503 to −461), respectively.

As shown in Fig. 4, nuclear proteins from pine cotyledons were able to bind GS1a AT boxes. Two shifts in the mobility of AT-1 box were detectable (Fig. 4, AT-1 panel): a strong signal corresponding to the slower migrating complex and a weaker faster migrating band. Both retardation complexes were abolished by adding increasing amounts of poly dA dT and the cold 60 bp DNA fragment in 100-fold excess. Two protein/DNA complexes were also observed when nuclear extracts were incubated with the 42 bp AT-2 box (Fig. 4, AT-2 panel). The abundance of the slower migrating shift was also affected by poly dAdT and completely abolished by the addition of 100-fold molar excess of unlabelled AT-2 DNA fragment. In contrast, these treatments did not alter the formation of the faster migrating complex to a great extent, indicating it could be a complex of unspecific nature. To investigate whether or not the nuclear factors involved in the retarded complex observed with AT-1 and AT-2 probes could be of proteinaceous nature, incubations with proteinase K of pine nuclear extracts were performed prior to the EMSA assays. The nuclear extracts were incubated in the presence of the enzyme for 10 min at 25°C, immediately before the binding assay. As depicted in Fig. 4, the treatment of nuclear extracts with proteinase K prevented the formation of the AT-1 retarded complexes. When the same type of experiments were conducted with the AT-2 element, binding activity was considerably reduced and the remaining formed complex changed its mobility. These findings indicate that pine nuclear proteins are involved in the formation of AT-1 and AT-2 complexes.

Since there are several examples of DNA binding proteins that are modified by phosphorylation (Sorger et al. 1987, Sorger and Pelham 1988, Datta and Cashmore 1989, Sarokin and Chua 1992), gel shift assays were

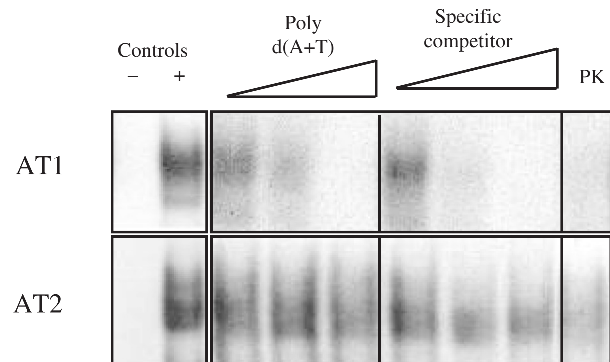

Fig. 4. Electrophoretic mobility assays showing binding of pine nuclear proteins to *GS1a* AT-rich boxes. Protein binding to DNA targets AT-1 (−720 to −661) and AT-2 (−503 to −461) boxes was analysed by electrophoretic mobility assays. The amount of protein added in each reaction was 4 µg of nuclear proteins. Unspecific (poly dA-dT) and specific competitors (cold AT-1 and AT-2 boxes) were included in the range of concentrations indicated in the Materials and methods. Increasing concentrations of competitor are indicated by the triangle. In the Proteinase K assay (PK), the proteins were treated with 3 µg of Proteinase K for 10 min, at 25°C previously to the binding reaction. All samples contained 2 µg of herring sperm DNA.

performed following alkaline phosphatase treatment of the extract. The results of these experiments are shown in Fig. 5. Treatment of nuclear extracts with calf intestine alkaline phosphatase prior to incubation with labelled AT-1 probe decreased the binding intensity of the DNA-protein complex, but had no effect on the mobility of the complex. The effect was abolished when NaF, an inhibitor of alkaline phosphatase, was also included in the alkaline phosphatase treatment reaction. This finding could be explained in terms of a change in the phosphorylation of the proteins involved in the formation of the AT-1 protein complex. This suggests that phosphorylation status of the proteins involved in the complex is important for their binding ability and thus seems to modify the affinity for the target DNA. Phosphorylated nuclear factors have been reported to be required for light responsive transcription of *rbcS* and *Lhcb* genes (Datta and Cashmore 1989) where an AT-rich sequence in the upstream region of both genes was present. Similar experiments were conducted with the AT-2 fragment, but no alkaline phosphatase dependent effect was observed (data not shown).

Several divalent cations, including Ba^{2+}, are known to promote DNA bending, while others such as Na^{+} do not have this property (Laundon and Griffith 1987, Perez-Martin and Espinosa 1994). These conformational changes could be induced by proteins interacting directly with their target DNA (Solano et al. 1995) and mostly have been shown to facilitate or to repress DNA–protein or protein–protein interactions involved in the transcriptional complex formation. In order to determine whether conformational changes occurred when nuclear proteins bound to AT-1, EMSAs were conducted in the presence of divalent cations, which uncover such distortions (Fig. 6). Both Mg^{2+} and Ba^{2+} enhanced the retardation of the shifted band thorough the gel. Enhanced shifts were not observed when the monovalent cation Na^{+} was used in the shift. These results suggest that pine nuclear proteins involved in the complex are able to induce conformational changes that stabilize the complex.

Taking into account transient expression results together with EMSAs, there are two consequences that could be inferred from that: (1) the deletion of the poly CT element located in the transcribed but untranslated region of the promoter seems to be critical for its function, even if most of the 5′upstream region is present; and (2) there are two A/T stretches localized in the most distal part of this promoter that bind nuclear factors of proteinaceous nature that could interact positively with the CT element to give full expression of the gene. These observed effects are illustrated in the model represented in Fig. 7. Interestingly, the simultaneous deletion of both types of boxes, A/T and CT, construct pGS6 gives a residual expression representing about 50% of the total promoter activity. These data reinforce the importance of the region containing the CT element in the control of gene expression and there are several examples of plant genes regulated by DNA sequences within the transcribed region (McElroy et al. 1990, Fu et al. 1995). Furthermore, the presence of *cis*-acting elements in the 5′ leader of Fed-1 mRNA was reported to regulate the light-induced increase in transcript abundance (Dickey et al. 1992). These sequences might include specific light regulatory elements or provide an environment in which the leader element can function normally. The poly CT fragment of the *GS1a* gene could hold *cis* elements that are able to interact with transcription factors themselves but that proved not to be the case, at least in the experimental conditions tested in this work. Therefore, according to our results, we propose that it can interact with sequences upstream either in the distal part of the promoter (A/T stretches) or the proximal region (−427 to −1) (Fig. 7), since when the CT and A/T-rich sequences were deleted the promoter activity was significantly restored.

In summary, we report here for the first time the functional characterization of regulatory elements involved in the transcriptional regulation of *GS1a*, a key gene involved in nitrogen metabolism of conifers. Our results indicate that the interaction of different *cis*-acting sequences located in separate regions of the promoter is required for gene transcription. Work is in

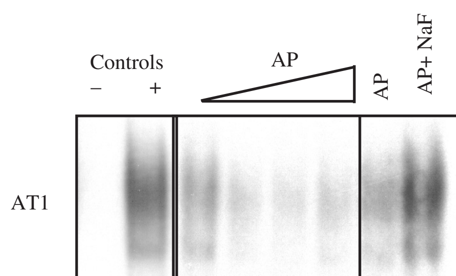

Fig. 5. Phosphatase treatment of binding reactions. Increasing amounts of alkaline phosphatase (AP) were included in the binding mixes, 0.7, 1.6, 2 and 2.6 μg of the enzyme, respectively. The free probe (AT-1 box) without protein is designed control (–) and the control (+) is the standard reaction with nuclear proteins. The right panel of Figure 5 indicates the effect of NaF in phosphorylation of nuclear proteins present in nuclear crude extracts involved in binding complex with AT-1 box. The assays were: AP, 1.6 μg of alkaline phosphatase and 0 NaF; AP + NaF, 1.6 μg of alkaline phosphatase and 50 m*M* NaF.

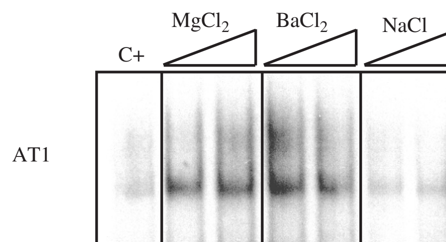

Fig. 6. Effect of cations in electrophoretic mobility shift assays. The chloride salt of the cations Ba^{2+}, Mg^{2+} and Na^{2+} was added at two different concentrations, 50 and 100 m*M*, respectively. The standard binding reaction mixture is designated as C + 0.

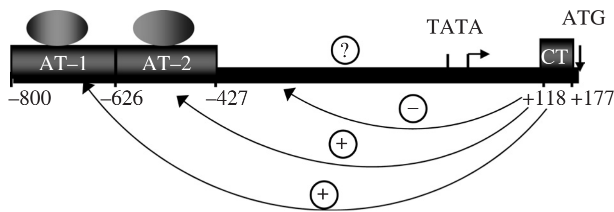

Fig. 7. Schematic representation of the proposed model for transcriptional regulation of the *GS1a* promoter. Boxes containing the putative *cis* elements span the sequences used for gel retardation assays. Light grey circles indicate the putative nuclear factors interacting with them. Relative activity of the gene is indicated by the + signs. No gene activity (–).

progress to characterize the nuclear *trans*-acting factors and to elucidate the molecular mechanisms involved in the relationships between A/T, CT and the minimal promoter.

Acknowledgements – This research was supported by a Grant from the Ministerio de Ciencia y Tecnología (BMC2003-04772) to FMC.

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Edited by J. K. Schjørring