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Original article

Structural and functional characterization of the 5' upstream region of a glutamine synthetase gene from Scots pine

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Abstract – We report here the isolation and characterization of a genomic clone encoding Scots pine (*P. sylvestris*) cytosolic glutamine synthetase *GS1a*. The clone contains the 5' half of the gene including part of the coding region organized in seven exons, interrupted by 6 introns and 980 bp upstream of the translation initiation codon. Earlier experiments carried out in our lab have shown that the *GS1a* gene is expressed in a light dependent fashion during the initial stages of Scots pine development. These data suggest a specific role for *GS1a* in ammonia assimilation in photosynthetic tissues of pine seedlings similar to the physiological role of GS2 in angiosperms. We have used a transcriptional fusion to *uidA* to transform pine cotyledons and *Arabidopsis* and demonstrated the ability of this 5'-upstream sequence to drive gene expression in both species and light regulation in *Arabidopsis.*

cytosolic glutamine synthetase / conifer / gene expression / N metabolism

Résumé – Caractérisation structurale et fonctionnelle de la région 5' du gène de la glutamine synthetase du pin sylvestre. Un clone génomique codant la glutamine synthetase cytosolique *GS1a* de pin sylvestre (*P. sylvestris*) a été isolé et caractérisé. Ce clone contient la moitié 5' du gène comprenant une partie de la séquence codante organisée en 7 exons séparés par 6 introns et également une séquence de 980 pb en amont du codon d'initiation de la traduction. Des expériences préliminaires menées dans notre laboratoire ont montré que la lumière régule l'expression du gène *GS1a* pendant les étapes initiales du développement du pin sylvestre. Ces données suggèrent un rôle spécifique de *GS1a* dans l'assimilation des ions ammonium par les tissus photosynthétiques des plantules de pin analogue au rôle physiologique de GS2 chez les angiospermes. Nous avons préparé une fusion transcriptionnelle avec le gène *uidA* pour transformer des cotylédons de pins ainsi qu'*Arabidopsis*. Nous avons ainsi démontré la capacité de cette séquence 5' de 980 pb à diriger (1) l'expression du gène chez ces deux espèces et (2) sa régulation par la lumière chez *Arabidopsis.*

glutamine synthetase cytosolique / conifère / expression génique / métabolisme de l'azote

1. INTRODUCTION

Glutamine synthetase (GS) plays a central role in nitrogen metabolism of higher plants. GS is responsible for the primary assimilation of ammonia produced by nitrate reduction or fixation of dinitrogen as well as the reassimilation of ammonia 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 [10]. As occurs in angiosperms it seems that a small multigene family could be operative in gymnosperms [12]. In the last years our studies have focused on

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ammonia assimilation in pine and studying regulation of the genes involved in the process [7]. Two distinct but homologous nuclear genes for GS have been detected and colocalized in the pine genome [2] both of them encoding cytosolic isoforms in conifers, *GS1a* and GS1b [1], but differentially expressed in pine seedlings [3].

Molecular data derived from the characterization of a *GS1a* cDNA clone showed that the gene is actively expressed in chloroplast containing tissues of developing seedlings and the level of the transcript was affected by developmental and light conditions [9]. Here, we present the DNA sequence of a partial genomic clone containing 7 exons of the coding region of *GS1a* gene. The clone includes 980 bp upstream of the functional ATG. So far, very few studies involving genomic clones from gymnosperms have been reported in the literature [4,16,18], and none of them correspond to nitrogen metabolism. We have studied the promoter activity of the 5'-untranslated region using fusions with the reporter gene *uidA* and the presence of DNA-protein interactions in the 5'flanking region of *GS1a* gene from Scots pine.

2. MATERIALS AND METHODS

2.1. Isolation of a genomic clone containing GS1 sequences in pine

Scots pine genomic DNA was digested with *Eco*RI and size fractionated by electrophoresis. Fragments were ligated to λgt10 and recombinant clones containing the *GS1a* gene were identified by screening using the 5'end of the cDNA clone previously isolated [8].

The fragment released from one of these clones by enzyme digestion was subcloned into the plasmid pGEM-3Z to generate the clone pGS217 and used for analysis and sequencing.

2.2. Fusions of the 5'region of *GS1a* to the GUS reporter gene

The 981 bp sequence upstream of the translation codon was isolated from the clone by *Hae III* digestion. The resulting fragment was subcloned into the vector pBI101 [15] creating a GS1: *uidA* gene fusion. The GS1 T-DNA construct, and also two controls, which were the original plasmid pBI121 containing the CaMV 35S promoter and pBI101, a plasmid containing a promoter-less 1.87 Kb GUS cassette in the binary vector pBin19, were transformed individually in *Agrobacterium tumefaciens* LBA4104.

2.3. Transient and stable transformation with the gene constructs

A Biolistic PDS-1000/He apparatus from Bio-Rad was used for particle bombardment of *P. pinea* cotyledons excised from embryos germinated for one day. After bombardment, cotyledons were maintained in the same medium where they were bombarded until GUS assays were performed 24 h after as described before [19].

For stable transformation, *Arabidopsis thaliana* WS ecotype plants were grown at 24 °C under a 16 h light/8 h dark regime and vacuum infiltrated as is described elsewhere [5]. T1 seeds were harvested in bulk and transformed seeds were selected in MS plates containing 50 μg mL^{-1} kanamycin. T2 seeds were harvested individually and kept for further analysis.

Histochemical GUS assays in bombarded cotyledons were performed as described by Rey et al. [19] whereas the fluorometric assay of gus in extracts of transgenic *Arabidopsis* were performed as described by Jefferson [14]. A 35S-promoter derivative pBI121 and promoter-less pBI101 plasmids were used as controls.

2.4. Gel retardation analysis

A DNA fragment used for gel retardation analysis containing a sequence from the 5'-untranslated region of *GS1a* was obtained by cleavage with restriction enzymes of the genomic clone pGS217. The fragment containing the A/T– rich region of 173 bp long was electrophoresed in 5% acrylamide gels excised and eluted by diffusion into 0.5 M NH_4OAc. 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 DNA (1–2 ng) labeled by filling in reaction with Klenow was incubated with 4 μg of crude nuclear extract as a source of protein as described previously [11]. Mixes were incubated for 30 min on ice. In non specific competition experiments 0 to 0.5 μg of poly dI-dC was also included in the mixes. 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. Gels were run in the cold room at 10 V cm^{-1} for 2–5 h.

3. RESULTS AND DISCUSSION

3.1. Sequencing and structural characteristics of the pine GS1 genomic clone

A λgt10 subgenomic library of Scots pine was screened for GS clones using the previously isolated pGSP114 pine GS cDNA [8]. About 1×10^6 recombinant clones were screened and four positives were isolated. One of these, pGS217 was subcloned and further characterized. As an initial step the genomic clone was entirely sequenced and determined to be 2543 bp in length. The comparison of nucleotide sequences between the gene and the cDNA [8] showed that the fragment contained the 5' half of the gene including part of the coding region organized in seven exons, interrupted by 6 introns and 980 bp upstream the translation initiation codon (*figure 1*).

The sizes of introns in the GS1 genomic clone were between 91 bp and 282 bp as shown in *table I*, all of them having the usual range size for angiosperm introns, which are typically shorter than most mammalian introns [23]. The AT percentage in higher plants introns is usually between 70% described for dicot plants and around 60% for monocot plants [22]. Unfortunately not many data are available for gymnosperm genes, however the introns in the GS1 clone showed an average AT percentage around 64%, which is within the range reported for angiosperms. We have also analyzed the sequences of 5'and 3'splice sites in all 6 pine cytosolic GS

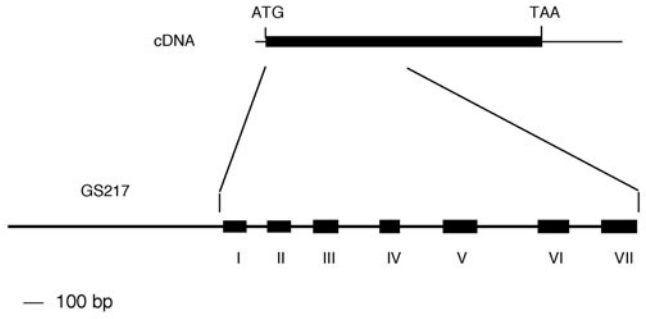

Figure 1. Comparative diagram of the pGS217 genomic clone and the full-length cDNA corresponding to the *GS1a* clone. Closed boxes represent coding regions. Exons are denoted by roman numbers from I to VII. Untranslated regions including introns are represented by a bar. The nucleotide sequence data reported will appear in the EMBL data bank under the accession number AJ 225121.

Table I. Characteristics of introns in the pGS217 pine genomic clone.

Intron nº	Size (bp)	(%) A/T	(%) Pyrimidines
1	107	62.6	40
2	91	63.7	65
3	282	69.8	50
4	96	63.5	40
5	236	60.4	70
6	151	63.6	60

introns and compared them with monocot and dicot plants, yeast consensus and vertebrate splice consensus sequences. The strict requirement in both sides of the intron for: G/GT in the 5'site and AG/G in the 3'end indicates a general use in all compared organisms.

We have also analyzed the presence of putative elements in the 5' region of the gene. There is a canonical TATA box at –35 bp from the transcription start site and a putative CAAT box at –138 bp. The 5' region also contains two A/T-rich sequences starting at –720 and –540 and 173 and 190 bp long respectively.

3.2. GUS expression in pine cotyledons and transgenic *Arabidopsis*

A transcriptional construct (C1) containing the complete 980 bp upstream the translation initiation codon fused to the GUS gene was created. The chimeric gene was used to test transient expression in *P. pinea* cotyledons. According to GUS histochemical assays, the 5'upstream region of the pine gene was able to drive gene expression in pine cotyledons. To further characterize the function of the 5'upstream region of the pine *GS1a* gene, stable GUS expression was studied in transformed *Arabidopsis* plants. Expression of the reporter

Table II. Effect of ammonium, light and dark treatments on GUS expression in transgenic Arabidopsis grown at 24 ºC under a 16 h light/8 h dark regime. Plants at the rosette stage were used. GUS activity was undetectable in roots and only data from the shoot apex are showed. Activities of C1 plants from 7 independent transformed lines were determined individually. The average +/– SD data of at least 3 different experiments are shown. Plants grown in a 16–h light/8–h dark regime were transferred to a medium containing 10 mM NH_4Cl (C1/N), continous light (C1/light) or continuous dark (C1/dark) for 3 days. A promoter-less derivative pBI101 was used as control.

Sample	MU (pmol mg^{-1} protein min^{-1})
Control (–)	1.47
C1	31.6 +/–1.05
C1/N	24.6 +/–3.16
C1/light	60.9 +/–2.57
C1/dark	3.05 +/–0.75

gene was absent or very low at the seedling and rosette stages, but apparent in adult plants with floral stems. These data therefore show that the 5'upstream region of pine *GS1a* gene is able to drive gene expression in an heterologous system. Moreover, our results are consistent with the report of Kojima et al. [17] indicating that a pine gene promoter can be operative in angiosperms and therefore suggesting that transcriptional machinery is well conserved between angiosperms and gymnosperms.

GS1a abundance determined in pine seedlings was unchanged when they were supplied with either inorganic nitrogen, nitrate or ammonium [6], however illumination increased the amount of the GS1 transcript [9]. In order to determine whether or not the expression driven by the 980 bp sequence from the *GS1a* gene is affected by these external stimuli in an heterologous system, GUS activity was measured in seven C1 independent transgenic lines following either supply with ammonium or light/dark treatments. As shown in *table II*, no meaningful changes were observed in NH_4^+-treated plants with regard to controls. By contrast, GUS activity levels were highly influenced by light in close agreement with light-enhanced GS transcript abundance in pine cotyledons.

3.3. Analysis of DNA-protein interactions in the 5' region of *GS1a* gene from Scots pine

We have carried out an in vitro study of interactions between nuclear factors from Scots pine cotyledons and an A/T-rich sequence in the upstream region of *GS1a* gene using the technique of gel retardation analysis. The fragment was end labeled with ^{32}P and incubated with crude nuclear extracts from Scots pine cotyledons. The concentration of salmon sperm DNA and poly dI-dC needed to eliminate non specific binding was first established (2 µg and 0.5 µg per assay, respectively). The binding reactions were electrophoresed on acrylamide gels to resolve

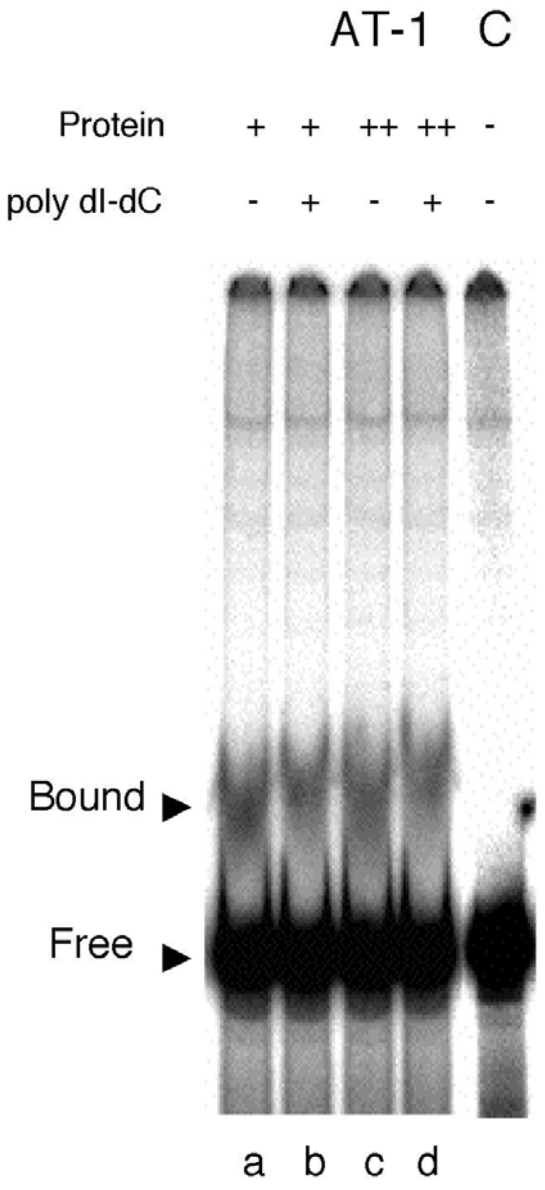

Figure 2. Gel retardation assay performed with cotyledon nuclear extracts and a restriction fragment containing the A/T-rich region starting at –720 bp in the upstream region of *GS1a* gene. Lanes a, b with protein nuclear extract (4 µg) and 0 or 0.5 µg of poly dI-dC respectively. Lanes c and d the same except 5 µg of protein nuclear extract was included. The C lane is a control without protein nuclear extract. All samples contained 2 µg of salmon sperm DNA.

the DNA-protein complexes from unbound DNA. *Figure 2* presents the results obtained in the gel retardation assay. The 173 bp long AT–1 fragment (starting at –720 bp) formed a complex that migrated more slowly than free DNA and that was not seen in the absence of nuclear proteins.

The AT–1 fragment represents an A/T rich region similar to rbcS, chs and Lhcb genes previously described [13, 20, 21]. We have identified the presence of *cis* elements in a light responsible promoter of a conifer *GS1* gene, but still experimental work is necessary to characterize further if the putative *cis* elements present in AT–1 region are functionally involved in regulation of the *GS1a* gene expression by light.

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