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Differential regulation of two glutamine synthetase genes by a single Dof transcription factor

Marina Rueda-López, Remedios Crespillo, Francisco M. Cánovas and Concepción Ávila*

Departamento de Biología Molecular y Bioquímica, Instituto Andaluz de Biotecnología, Unidad Asociada UMA-CSIC, Campus Universitario de Teatinos, Universidad de Málaga, 29071 Málaga, Spain

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*For correspondence (fax +34 952 13 2376; e-mail cavila@uma.es).

Summary

The PpDof5 transcription factor from maritime pine (*Pinus pinaster)* is a regulator of the expression of glutamine synthetase (GS) genes in photosynthetic and non-photosynthetic tissues. PpDof5 mRNA is detected almost ubiquitously during pine development with low levels of gene expression in green tissues and much higher levels in roots and lignified shoots. The PpDof5 protein expressed in bacteria binds to oligonucleotide probes containing the AAAG core sequence derived from the promoters of *GS1a* and *GS1b* genes. Transient expression experiments in agroinfiltrated tobacco leaves and in pine protoplasts demonstrated that PpDof5 is able to *trans*-regulate differentially the transcription of both *GS1a* and *GS1b*. PpDof5 activated transcription of the *GS1b* promoter and, in contrast, behaved as a transcriptional repressor of the *GS1a* promoter. These results support a regulatory mechanism for the transcriptional control of the spatial distribution of cytosolic GS isoforms in pine. Considering the precise expression patterns of GS1 genes required to fulfil the ammonium assimilation requirements during tree development, we hypothesize that PpDof5 could have a key role in the control of ammonium assimilation for glutamine biosynthesis in conifers. A regulatory model of GS1 gene expression in pine is proposed.

Keywords: ammonium assimilation, conifers, Dof factors, differential expression, glutamine synthetase, transcriptional regulation.

Introduction

Growth is a complex trait that can be subdivided into several components contributing to the final outcome. The growth components are under genetic control and they are regulated by different sets of genes. Nitrogen is quantitatively the most essential nutrient for plants and a major limiting factor in plant growth and productivity (Tabuchi *et al.*, 2007). A number of research efforts have centred on elucidation of the key steps of the nitrogen assimilation process, including nitrate uptake and reduction, incorporation of ammonium into glutamine and the biosynthesis of glutamate (Forde and Lea, 2007; Lea and Azevedo, 2006).

Therefore, an increased knowledge of nitrogen metabolism and the generation of trees having highly efficient nitrogen assimilation and metabolism are of broad interest to tree breeders, especially to decrease the cost/production ratio; this includes relevant issues such as (i) increase in yield; (ii) decrease in the pollution associated with the use of fertilizers; and (iii) improving the resistance of trees to different stresses (Gallardo *et al.*, 2003).

In conifer forests, low soil pH, high residual content of lignin and other secondary plant products in the soil limit nitrification. Consequently, ammonium is the predominant source of nitrogen for tree nutrition. Therefore, primary ammonium assimilation from the soil is a crucial process for tree growth and development. However, the internal recycling of nitrogen is particularly necessary to maintain the nitrogen economy of the tree since in many woody plants nitrogen assimilated from the soil may not be used immediately but is stored for use during the next growing season (Suarez *et al.*, 2002). In conifers seasonal nitrogen economy involves efficient regulation of glutamine metabolism and its interconversion into other nitrogen compounds.

In higher plants, all nitrogen, whether derived from nitrate, ammonium, nitrogen fixation or generated by other

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reactions that release ammonium within the plant, is channelled through glutamine synthetase (GS; EC 6.3.1.2). Glutamine synthetase catalyses the incorporation of ammonium into the amide group of glutamine (Hirel and Lea, 2001). Recent genetic and molecular approaches have shown that GS may be a key component of efficiency of nitrogen use and yield (Hirel *et al.*, 2001; Man *et al.*, 2005; Martin *et al.*, 2006; Tabuchi *et al.*, 2005). In conifer species, the reaction catalyzed by cytosolic glutamine synthetase (GS1) is a key step controlling the plant's capacity to assimilate and recycle nitrogen. Two different genes, *GS1a* and *GS1b*, encode GS1 isoforms in pine, and the isoforms exhibit distinct molecular and kinetic properties (Avila *et al.*, 1998; Avila-Sáez *et al.*, 2000; de la Torre *et al.*, 2002). Both genes display overlapping yet distinct patterns of expression. *GS1a* is expressed almost exclusively in the chlorophyllous parenchyma of photosynthetic tissues, whereas *GS1b* is expressed in vascular cells throughout the plant (Avila *et al.*, 2001a). According to these findings different roles for have been proposed these proteins. Expression of *GS1a* would be associated with assimilation of ammonium in photosynthetic cells, whereas expression of *GS1b* in the vascular bundles is probably associated with nitrogen transport and translocation (Avila *et al.*, 2001a).

Mechanisms that underlie differential expression of GS isoforms in photosynthetic and vascular cells involve the interaction between tissue-specific transcriptional factors and functional motifs in the promoter regions of both genes. The existence of AT-rich repeated sequences in the *GS1a* promoter similar to those in light-regulated genes such as *rbcS*, *cab* and *GLN2* have been previously described (Avila *et al.*, 2001b). Functional characterization studies have confirmed the existence of regulatory sequences in the upstream region of the gene that interact with *trans*-acting factors essential for transcriptional activity (Gómez-Maldonado *et al.*, 2004a), though the identity of such factors needs to be determined.

Phenylpropanoid metabolism in lignifying cells of the vascular elements releases important amounts of ammonium that must be efficiently reassimilated by GS in order to avoid nitrogen deficiency (van Heerden *et al.*, 1996; Razal *et al.*, 1996; Singh *et al.*, 1998). The *GS1b* gene, expressed in vascular cells, has been proposed to play a key role in recovering and reassimilating this released ammonium (Cantón *et al.*, 2005; Gómez-Maldonado *et al.*, 2004c). In pine, two members of the R2R3-MYB family were shown to bind regulatory elements in the *GS1b* promoter containing common motifs found in the promoters for enzymes involved in lignin biosynthesis (Gómez-Maldonado *et al.*, 2004c). These Myb proteins act like transcriptional activators and the findings suggest a simple mechanism to coordinate lignin biosynthesis and the reassimilation of ammonium in the same cell types via transcriptional regulation.

To extend our understanding of the molecular mechanisms of transcriptional control of ammonium assimilation in pine, we have identified several putative *cis*-acting elements in *GS1a* and *GS1b* promoters that could interact with Dof (DNA binding with one finger) factors. The Dof proteins are members of a major family of plant transcription factors that play diverse roles in regulating gene expression (Yanagisawa, 2004). Recently, metabolically engineered Arabidopsis plants expressing Dof1 from maize showed improved nitrogen assimilation and growth under low-nitrogen conditions, suggesting that Dof1 could be a key factor in coordinated gene expression involved in carbon-skeleton production required for assimilation of nitrogen into the plant (Yanagisawa *et al.*, 2004).

Interestingly, it has recently been reported that Dof factors might also be involved in the regulation of lignin production (Rogers *et al.*, 2005). Microarray experiments determined the pattern of transcript abundance of Dof family members to be consistent with a role in lignin accumulation. This suggests that Dof family members might be involved in the regulation of genes in the lignin toolbox under conditions resulting in increased lignin deposition.

In the present study, we report the isolation and characterization of a novel Dof-encoding gene in maritime pine, *Pinus pinaster* (*PpDof5*). This transcription factor is highly expressed in germinating seedlings as well as in developing trees where the transcripts are mainly present in non-photosynthetic tissues with a pattern of expression parallel to *GS1b*. PpDof5 protein is localized in the nuclei, as expected for a transcription factor. Protein–DNA interaction experiments have shown that the recombinant Dof protein was able to bind *in vitro* to sequences defined as the core motif required for binding of transcription factors with one finger (Dof) class (Yanagisawa, 1996) in both *GS1a* and *GS1b* native promoters. Transient expression studies in agroinfiltrated tobacco leaves and pine protoplasts have shown that PpDof5 has antagonistic roles in the *trans*-regulation of both *GS1a* and *GS1b* promoters and the regulatory regions have been identified by functional deletion analyses. We conclude that PpDof5 is a transcriptional activator of *GS1b* while it behaves as repressor of *GS1a*. The role of PpDof5 in the regulation of pine GS expression as well as its implication in the regulation of nitrogen metabolism in conifers is discussed.

Results

*Identification of a Dof-encoding gene (*PpDof5*) from maritime pine*

A large-scale expressed sequence tag (EST) sequencing project for maritime pine (Cantón *et al.*, 2003) has created an EST database from different woody tissues (Dantec *et al.*, 2004). The aim of this project was the analysis of the

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transcriptome of wood formation in maritime pine. With this goal a composite cDNA library was made with mRNA isolated from normal, compression, opposite, early and late wood of maritime pine uni-directionally cloned into Uni-ZAP XR from Stratagene (http://www.stratagene.com/). From the library a cDNA clone (PP100A03) harbouring a 1.7-kb insert was completely sequenced and shown to contain a nearly full-length cDNA designated *PpDof5* (EMBL nucleotide sequence database accession number AM884254). The *PpDof5* sequence contained a 5′ untranslated region of 126 nucleotides, an open reading frame (ORF) encoding a polypeptide of 356 amino acids and a 3′ non-coding region of 567 nucleotides ending in a short poly(A) tail. BLAST analysis performed with the full-length sequence of *PpDof5* revealed near identity between the *P. pinaster* sequence and an EST (TC66796) from loblolly pine (*Pinus taeda)* (http://www.tigr.org/tdb/e2k1/pine/index.shtml) that has been named *PtDof5* (Shigyo *et al.*, 2007). Since both cDNA sequences are almost identical we infer that both correspond to the same protein in both pine species. According to these findings we refer the *P. pinaster* cDNA as *PpDof5*.

PpDof5 *encodes a polypeptide that is related phylogenetically to ancestral Dof factors*

Figure 1(a) shows that the deduced PpDof5 polypeptide contains a highly conserved Dof domain in the N-terminal region and a putative nuclear localization signal (NLS) adjacent to the Dof domain: RRSKPHPTQVQ. The C-terminal region of the protein is highly divergent in sequence and should contain a regulatory domain as functionally determined for other Dof transcription factors (Yanagisawa, 2002). To examine the phylogenetic relationships of the PpDof5 sequence with Dof proteins from angiosperms, an unrooted phylogenetic tree was constructed. We have included in this study the sequences of Dof genes from three species whose genomes have been completely sequenced: a dicotyledoneous angiosperm (*Arabidopsis thaliana*), a monocotyledon (*Oryza sativa*) and a woody angiosperm (*Populus trichocarpa*). The tree was constructed using the very well conserved sequence corresponding to the Dof domain. The complete tree, including all Dof genes from the three species as well as the pine *PpDof5* can be found as

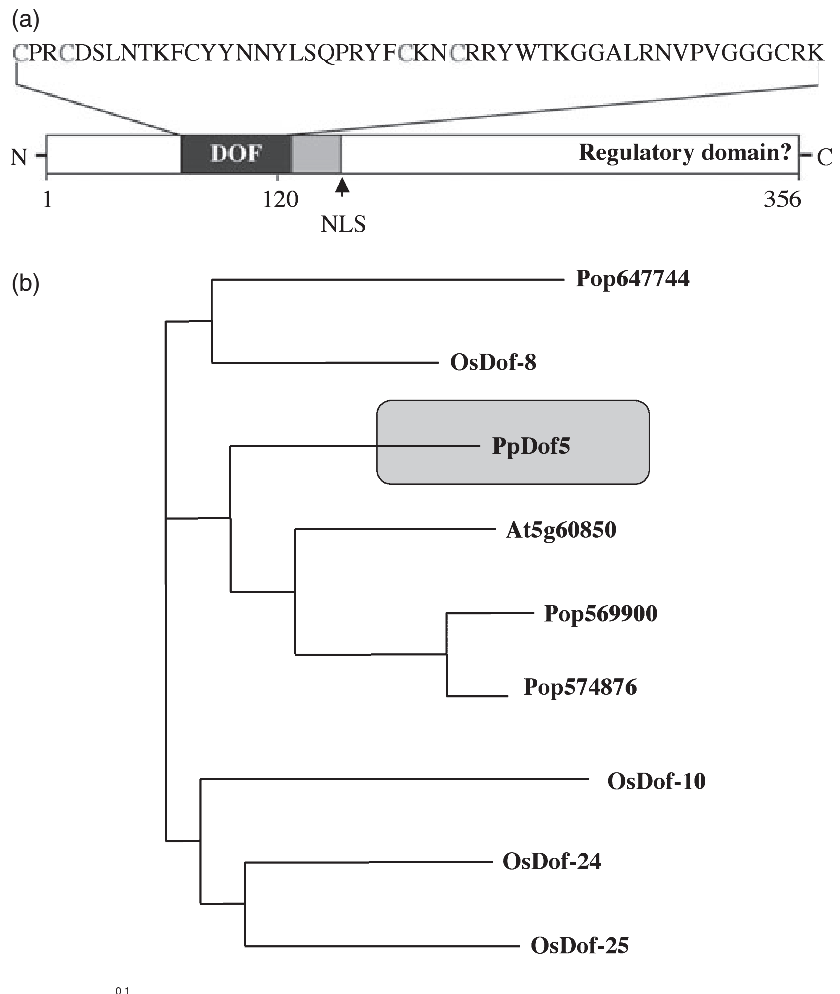

Figure 1. Phylogenetic tree of Dof proteins from different organisms.
A phylogenetic tree was constructed using the neighbour-joining method of the CLUSTALW program (Thompson *et al.*, 1994). The alignment was performed using a common homologous region spanning the 52 amino acids of the Dof domain of 102 Dof sequences from Arabidopsis, rice and poplar as well as the pine sequence. Abbreviations correspond to: At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Pop, *Populus trichocarpa*; Pp, *Pinus pinaster* genes. The whole tree is presented in the Supplementary Material as Figure S1. A cluster with the closest related genes to PpDof5 is represented.

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Supplementary Material (Figure S1). Figure 1(b) represents a tree including the closest genes from the three species together with the pine gene. A previous phylogenetic study of Dof genes, including from rice, Arabidopsis and barley, classified Dof proteins into seven subfamilies (Moreno-Risueño *et al*., 2007). According to this classification the cluster depicted in Figure 1(b) would be included in the subfamily B, where the presence of conserved motifs characteristic of the subfamily indicated that the members originated before the divergence of the angiosperm and the gymnosperm ancestors.

The PpDof5 *gene is ubiquitously expressed in pine tissues*

To explore the pattern of expression of *PpDof5* during tree development, total RNA was isolated from mature dry embryos, embedded embryos and germinating embryos (2 days after imbibition (DAI)). At the seedling stage (2.5 cm cotyledon length) three kind of samples were taken: cotyledons, hypocotyls and roots. We also analysed expression of *PpDof5* in samples of needles, lignified shoots and roots taken from 1-year-old trees. Since accumulation of *PpDof5* transcript was difficult to detect by northern blot assay (data not shown), semiquantitative RT-PCR analysis was performed. As shown in Figure 2(a), *PpDof5* transcripts were detected in all samples analyzed, although the level of expression was lower in dry embryo (DE) where it was almost undetectable and the needles of 1-year-old trees. We analysed in parallel the level of expression of *GS1a* and *GS1b* in the same samples (Avila *et al*., 2001a). As previously shown, *GS1b* transcript levels were almost constant in the three embryonic stages considered, whereas *GS1a* transcript levels increased through the embedding and germination of seeds. In developing seedlings, *GS1a* expression was almost exclusively located in the green tissues with very low levels of transcript expression in roots. In cotyledons and needles, *GS1b* expression decreased with the age, in an opposite manner to *GS1a* transcript expression that was undetectable in roots of 1-year-old trees.

The observed pattern of expression of *PpDof5* transcripts suggests a predominant role of this transcription factor in non-green tissues as occurs with the *GS1b* gene. Figure 2(a) shows the levels of transcripts for *eiF4-A* determined for all developmental stages as an internal control. The levels of transcripts normalized against the *eiF-4A* control are shown in Figure 2(b).

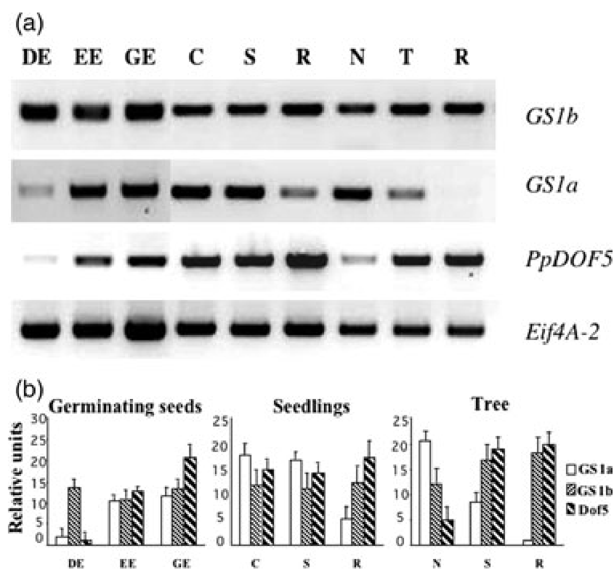

Figure 2. A RT-PCR analysis of PpDof5 expression in pine tissues.
(a) Total RNA was isolated from dry embryos (DE), embedded embryos (EE) and germinated embryos (GE), at the seedling stage [cotyledons (C), shoots (S), roots (R) of seedlings with cotyledons 2.5 cm in length] and from a 1-year-old tree [needles (N), lignified shoots (T) and roots (R)]. Reverse transcription of RNA was performed in the presence of oligodT oligonucleotide. The first-strand cDNA was amplified by PCR using specific primers for *GS1a*, *GS1b* and *PpDof5* transcripts. *EiF4A-2* was used as the internal control.
(b) Relative transcript levels normalized to the internal control (*Eif4A-2*). Three replicates of each experiment were made. Standard errors are indicated.

The pine PpDof5 protein is targeted to the nucleus

To investigate the subcellular location of the PpDof5 protein *in vivo*, a transient expression system using onion epidermal cells was assayed. The full-size ORF of the *Dof5* gene was fused in frame to the green fluorescent protein (*GFP*) reporter gene under the transcriptional control of the *CaMV 35S* promoter. The fusion construct *35S::Dof5-GFP* was introduced into onion epidermal layers by particle bombardment. Microscopic observations indicated that the fluorescence of the GFP was targeted to the nuclei of cells bombarded with the constructs carrying the PpDof5 protein (Figure 3c,f). Therefore, as expected for a putative transcription factor, PpDof 5 was localized in the nucleus. The location of nuclei in onion epidermal cells was observed in bright field controls (Figure 3a,d) and with 4′-6-diamidino-2-phenylindole (DAPI) staining (Figure 3b,e).

PpDof5 regulates the expression of the GS1a *and* GS1b *promoters in agroinfiltrated tobacco leaves*

Because promoters containing the AAAG core sequence are likely to be the targets for Dof proteins (Yanagisawa, 2004), we addressed the question of whether PpDof5 modulates the transcriptional activity of *GS1a* and *GS1b* promoters by performing transient expression experiments *in planta* using infiltration of *Agrobacterium tumefaciens* cells into tobacco leaves.

A 981-bp sequence containing de *GS1a* promoter (Avila *et al*., 2001b) fused to the GUS reporter gene was used as the reporter construct (Figure 4a). As the effector we used the whole ORF of *PpDof5* under the control of the *CaMV 35S* promoter. A promoter-less *gusA* control plasmid was used as the negative control. Co-infiltration experiments in

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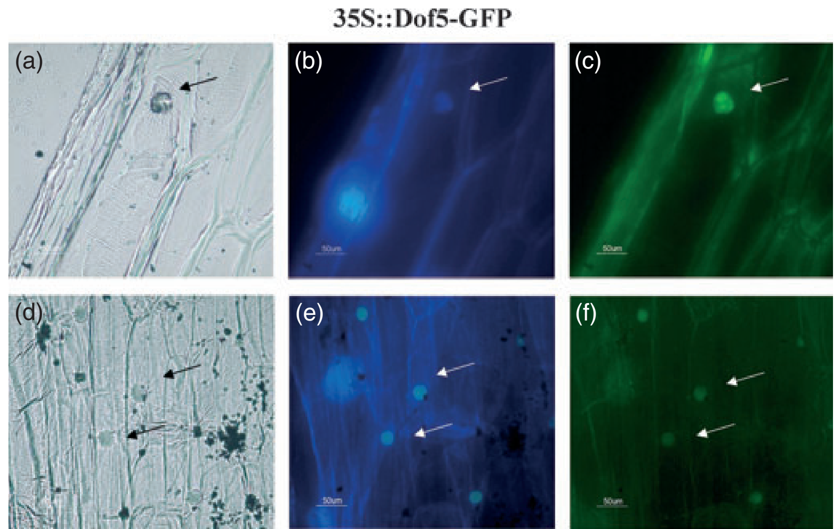

Figure 3. Subcellular location of PpDof5 in onion epidermal cells.
Epidermal onion cells were transiently transformed with *35S::PpDof5-GFP*. After incubation for 24 h, cells were observed under bright field (a,d). Detection of nuclei was achieved by staining with DAPI (b,e) and observed by fluorescence imaging (c,f). Arrows point to the location of the fluorescent nucleus where the fluorophore is reconstituted.

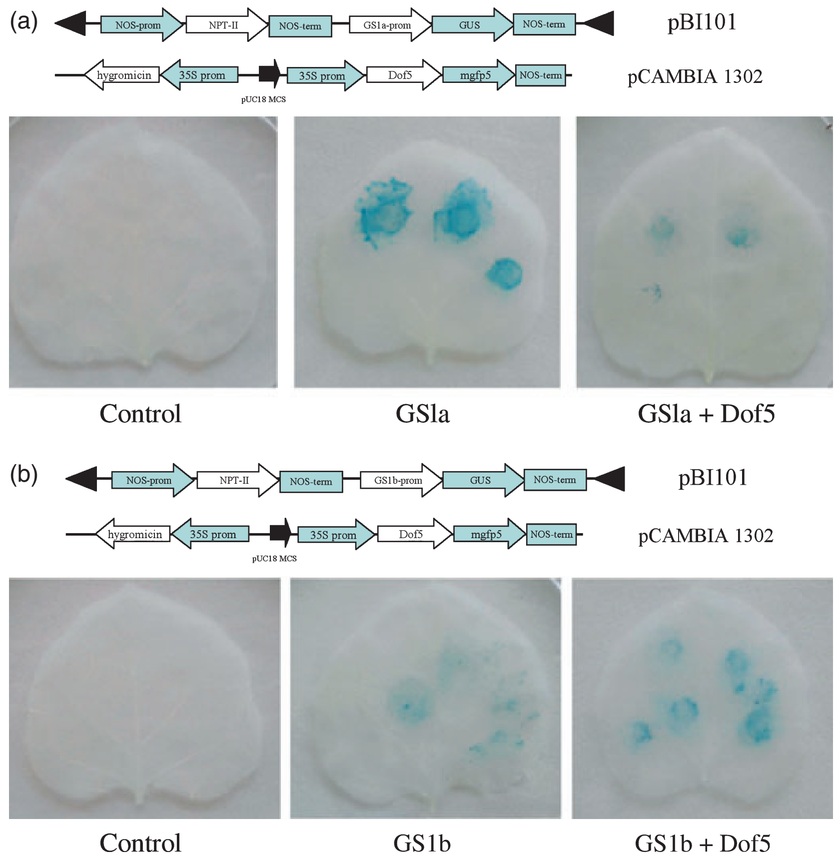

Figure 4. Transient GUS expression in tobacco leaves driven by *GS1a* and *GS1b* promoters.
(a) Schematic representation of reporter and effector constructs used in transient expression assays with the *GS1a* promoter and (b) with the *GS1b* promoter. *Agrobacterium tumefaciens*-mediated transient transformation was conducted on mid-size to near fully expanded leaves, still attached to 6- to 7-week-old intact plants. Bacterial suspensions were infiltrated into leaf mesophyll (Yang *et al.*, 2000). Histochemical assays were performed 2 days after infiltration. After GUS staining, plant tissues were fixed (5% v/v formaldehyde, 5% v/v acetic acid, 20% ethanol). Chlorophyll was extracted from the photosynthetic tissues with 70% v/v ethanol.

Nicotiana benthamiana leaves were performed essentially as described previously (Yang *et al.*, 2000). For transient co-expression experiments bacterial cultures were mixed in a 1:1 ratio.

As shown in Figure 4(a), infiltration of *GS1a::GUS* resulted in high level of GUS activity in the areas where *Agrobacterium* cells were applied. In contrast, co-expression of the reporter *GS1a::GUS* and *35S::Dof5* effector construct significantly lowered the GUS activity under the control of the *GS1a* promoter. These data provide evidence that PpDof5 functions as a negative regulator in the transient experiments.

We have also tested in tobacco leaves the effect of PpDof5 on the transcriptional activity of the *GS1b* promoter.

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A reporter construct containing the 1168-bp upstream sequence of the *GS1b* gene was fused to the GUS reporter gene. Co-expression experiments were performed as described before for the GS1a construct. As shown in Figure 4(b) the effector construct that contains PpDof5 increased the GUS activity under the control of the *GS1b* promoter, suggesting that PpDof5 may function as positive regulator of the gene.

PpDof 5 binds in vitro *to DOF motifs in the promoter of pine* GS1 *genes*

We were interested to determine whether the observed *in vivo* transcriptional regulation of *GS1* genes is supported by interactions between PpDof5 and the binding core AAAG present in the *GS1a* and *GS1b* promoters. Electrophoretic mobility shift assays (EMSA) were performed to evaluate whether PpDof5 was capable of specific binding *in vitro* to the DOF motif in the promoters of *GS1a* and *GS1b* genes,. The PpDof5 protein expressed as a polyHis fusion in *Escherichia coli* was incubated with labelled-oligonucleotide probes containing the Dof motif that were deduced from the corresponding region in the pine GS1 promoters. Although a number of probes were assayed, only those showing specific binding are presented.

The *GS1a* probe was shifted only when incubated with the PpDof5 protein and not when the protein was absent (Figure 5a, control). Two shifts were detectable: a strong signal corresponding to the faster-migrating complex and a weaker slower-migrating band. Therefore, on the basis of the number of bands that were observed, it would appear that there are two PpDof binding sites in the probe. The addition of increasing amounts of the unspecific competitor poly dI-dC gradually decreased the abundance of the faster-migrating complex, whereas the slower one

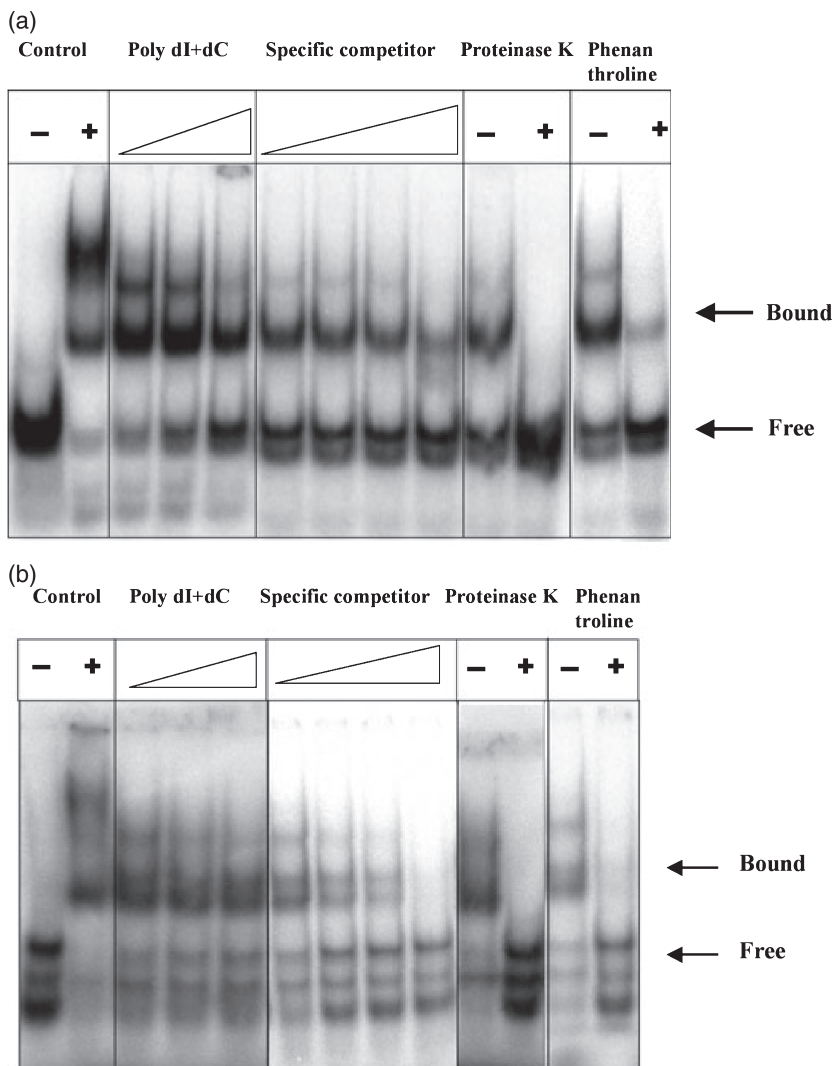

Figure 5. Electrophoretic mobility shift assays (EMSAs) of the recombinant PpDof5 with oligonucleotide probes derived from *GS1a* and *Gs1b* promoters.
(a) A probe of 44 bp derived from the *GS1a* promoter was ^{32}P labelled: 5′-AACTTTCTTTGTT-GAAGTGAGTATGAATGGCTTTCTAATTTGCA-3′. The probe without recombinant PpDof5 is designated control (–) and control (+) is the standard reaction with recombinant PpDof5 and without any competitor added. Competitions experiments were performed using increasing amounts either of the non-specific competitor (polydI-dC) or the unlabelled probe. Increasing molar amounts (10×, 20×, 50×, 100×) are indicated by the triangle. For the proteinase K and phenantroline experiments –/+ signs indicate with/without the respective treatment.
(b) A probe of 22 bp derived from the *GS1b* promoter was ^{32}P labelled as described previously: 5′-GTGAGGAAAAAAAGAAATTGGG-3′. The analyses of binding complexes were performed as described for *GS1a* promoter probe. Protein–DNA complexes are designated as ‘bound’ and the probe alone as ‘free’.

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was then more prominent. The addition of a molar excess of the unlabelled probe revealed that it effectively competes with the *cis*-elements in the *GS1a* promoter for PpDof5 binding (Figure 5a, specific competitor). The formation of the shifted bands were protein-dependent as the treatment of the *E. coli* expressed protein with proteinase K prior to the binding assay prevented the formation of the retarded complex (Figure 5a, proteinase K). Since it has been previously shown that the DNA-binding activity of the zinc finger in other Dof proteins was effectively inhibited by the metal-chelator 1,10-phenantroline (Mena *et al.*, 1998), we examined whether the binding activity using PpDof5 was also sensitive to this compound. When the PpDof 5 protein was incubated with the *GS1a* promoter probe in the presence of 5 mM 1,10-phenantroline binding was not detected (Figure 5a, phenantroline), as expected for a DNA-binding activity that depends on metal ions.

Parallel binding experiments were performed using the recombinant PpDof5 protein and a probe derived from *GS1b* promoter (Figure 5b). Three shifts in the mobility of the probe were apparent (Figure 5b, control). The retarded complexes were less efficiently competed by polydI-dC than by the cold probe that completely abolished PpDof binding. Similar to what we found in the EMSAs for the GS1a probe, the formation of the complexes was dependent on intact PpDof protein and they were also abolished in the presence of 1,10-phenantroline.

PpDof5 has an antagonistic regulatory function in the expression of GS1a and GS1b promoters in pine protoplasts

The functional relevance of the interaction observed *in vivo* in tobacco leaves between PpDof5 and the *GS1* promoters was further tested in plant cells by transient expression assays in pine protoplasts.

Figure 6(a) shows schematically the constructs used in the assays with the promoter of the *GS1a* gene. The effector construct contained the complete cDNA from *PpDof 5* controlled by two copies of *CaMV 35S* promoter. The reporter used in this experiment was previously described by Gómez-Maldonado *et al.* (2004a) and contained the –800 bp promoter region of *GS1a* fused to the GUS-reporter gene and the nos terminator. Pine protoplasts were transiently transformed by electroporation with the reporter alone or in combination with the effector at a 1:1 molar ratio. As shown in the Figure 6(b) the co-expression of the reporter with the effector lowered the GUS activity under control of the *GS1a* promoter to less than a half of that found with the reporter alone. These data are consistent with the those observed in the agroinfiltration experiments of tobacco leaves and indicate that PpDof5 functions as a negative regulator of the GUS activity driven by the *GS1a* promoter.

We further investigated the *in vivo* interaction between PpDof5 with the *GS1b* promoter. The constructs are schematically represented in Figure 6(c). The effector construct used was as described before for transient expression

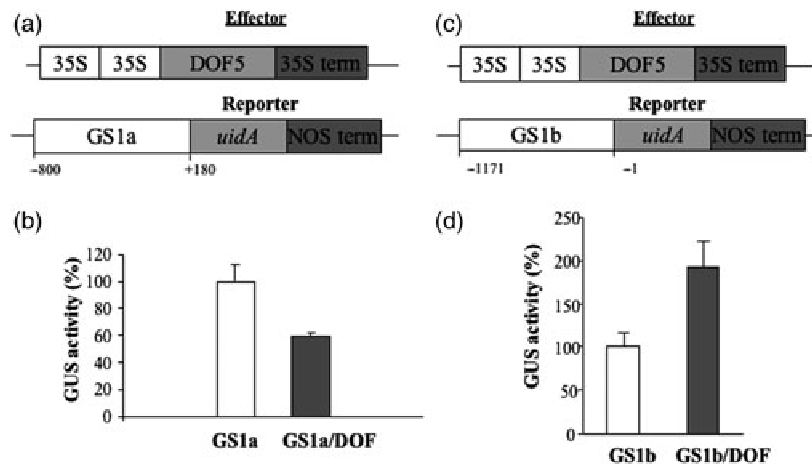

Figure 6. PpDof5-mediated regulation of the *GS1a* and *GS1b* promoters in pine protoplasts.
(a) Schematic structure of reporter and effector constructs used in the transient expression experiments for *GS1a*. The effector construct contained the complete ORF of the cDNA of PpDof5 under the control of two copies of *CaMV 35S*. A promoterless derivative was used as a negative control and the background level was subtracted for the calculation of GUS activity.
(b) Transient expression assays of pine protoplasts resulting from co-electroporation with a combination of reporter and effector plasmids at a 1:1 molar ratio. In each assay sets of four pine protoplasts were electroporated and three replicates of each experiment were done. Standard errors are indicated. GUS activity was expressed as percentage of the level observed for the expression with the reporter construct alone (0.14 nmol MU min^{-1} mg^{-1} proteins).
(c) Schematic structure of reporter and effector constructs used in the transcriptional activation assays of *GS1b*. The effector construct is the same as described for *GS1a*.
(d) Quantitative analysis of GUS activity in pine protoplasts. GUS activity was determined as described previously. After correction activity was expressed as percentage of the level observed with the reporter construct (0.15 nmol MU min^{-1} mg^{-1} proteins).

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studies with the *GS1a* promoter. The *GS1b* promoter fused to the GUS reporter gene was used as the reporter construct (Gómez-Maldonado *et al.*, 2004b) and the transient expression data are shown in Figure 6(d). The co-transfection of the *GS1b* promoter with PpDof5 as the effector resulted in increased GUS activity that turned out to be more than double the activity determined using the reporter alone. These results, together with those obtained in the agro-infiltration experiments, indicate that PpDof5 could be a *trans*-activator of the *GS1b* promoter in pine.

The functional interaction observed between PpDof 5 and GS genes was further studied using several deletions for either promoter (Figure 7). Transient expression assays of pine protoplasts were performed using the indicated combination of reporter and effector plasmids at a 1:1 molar ratio. As shown in Figure 7(a), the negative regulation caused by the transcription factor was abolished when the distal part of the *GS1a* promoter was absent (GS1a-P3 construct). Interestingly, the deleted sequence contains the binding sequence used for the shift assays shown in Figure 5(a). Similar experiments were performed with the *GS1b* promoter (Figure 7b). The activation of *GS1b* transcriptional activity caused by PpDof5 was completely suppressed after deletion of 768 bp of the promoter. This fragment contains the sequence used for the shift assays shown in Figure 5(b).

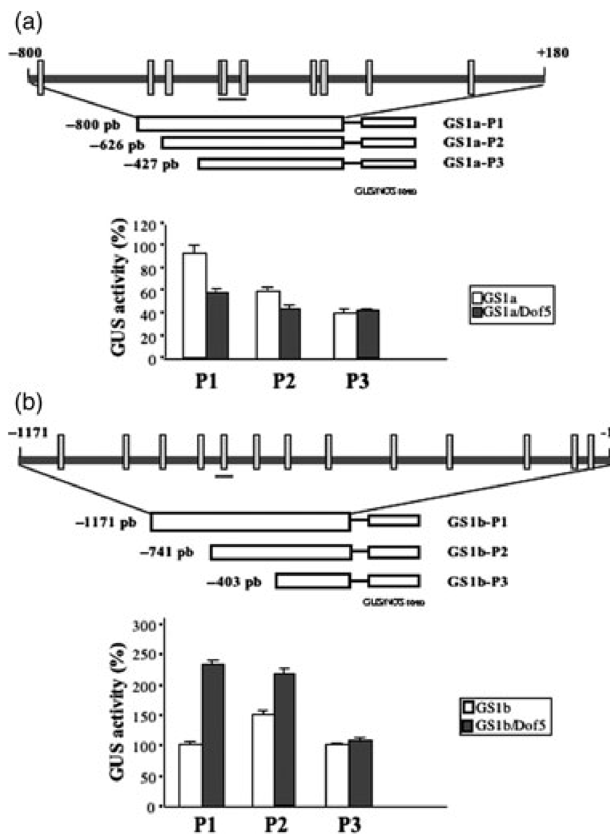

Figure 7. Functional deletion analysis of the *GS* promoters.
(a) Schematic representation of chimeric promoter *GS1a*-GUS constructs and transient expression assays in pine protoplasts.
(b) Schematic representation of chimeric promoter *GS1b*-GUS constructs. Numbers indicate the distance (in bp number) relative to the transcription start sites. The grey boxes indicate the putative Dof-binding sites found in *GS1a* and *GS1b* promoters. The underlined grey boxes indicate the position of the probes used in EMSA experiments. The effector constructs are the same for either gene, *GS1a* and *GS1b*, and are described in Figure 6. Each value of GUS activity represents the average of four independent protoplast electroporation events with GUS quantification by triplicate.

Discussion

The expression of *GS1a* and *GS1b* genes is controlled at transcriptional level with a precise space/time pattern, suggesting that they play different and non-redundant roles in nitrogen metabolism in conifers (Cánovas *et al.*, 2007; Suarez *et al.*, 2002). We found that two members of the R2R3 MYB family of transcription factors that are expressed in lignifying cells were able to bind the *GS1b* promoter (Gómez-Maldonado *et al.*, 2004c) and activate its transcriptional activity. Recent transcriptome analyses indicate that Dof factors might also be involved in regulating lignin production (Rogers *et al.*, 2005) and carbon–nitrogen interactions (Yanagisawa *et al.*, 2004). These results, together with the fact that both pine *GS1* promoters contain putative *cis* elements having the core sequence for binding Dof factors, prompted us to study the possible regulation of GS genes by Dof transcription factors. In order to further understand the regulatory mechanisms controlling the differential distribution of GS isoforms, we previously performed a search for binding sites of Dof factors in the promoter regions of pine and angiosperm *GS* genes (*A. thaliana* as an annual angiosperm model and *P. trichocarpa* as a woody angiosperm). The data shown in Table S1 suggest that the Dof factor may control transcription of *GS* genes in different plant species.

In this paper, novel insights into the transcriptional regulation of *GS* genes in pine are presented. We propose that the *PpDof5* gene has a role in determining the spatial distribution of GS1 isoforms throughout the plant. The Dof family is a particular class of zinc finger domain transcription factors characterized by a conserved region of 50 amino acids with a C2-C2 finger structure, associated with a basic region, that binds specifically to DNA sequences with a 5′-AAAG-3′ core. The Dof proteins typically consist of multiple domains, including a highly conserved N-terminal DNA-binding domain and a C-terminal domain for transcriptional regulation. The diversity of the remaining part of the protein outside the Dof domain reflects the variety of functions described for these Dof factors (Yanagisawa, 2002).

Recently Dof1 from maize, a transcription factor involved in the activation of several genes encoding enzymes associated with organic acid metabolism, has been overexpressed in *A. thaliana*, resulting in positive effects on nitrogen

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assimilation and growth (Yanagisawa *et al.*, 2004). We have analyzed the possibility that *PpDof5* could be an orthologue of the maize gene. The DNA-binding domains of the maize Dof1 and PpDof5 posses highly conserved regions, as is characteristic of Dof family of transcription factors. However, the C-terminus of both proteins was completely different, probably reflecting functional differences between them. *PpDof5* is closely related to *At5g60850*, *Pop569900* and *Pop574876* genes encoding putative Dof factors of unknown function in Arabidopsis and poplar.

The evolutionary study of the family of Dof transcription factors (Shigyo *et al.*, 2007) suggests that *Dof* genes, from diverse plant groups including mosses, algae and terrestrial plants, can be classified into three groups, A, B and C. PpDof5 could be assigned to group C which includes certain Arabidopsis genes tightly linked to vascular development (Konishi and Yanagisawa, 2007). These authors also suggest a correlation between the diversification of the group C-type Dof domain proteins and their involvement in organ development.

In addition, the phylogenetic analysis of the Dof family in a variety of representative species from green algae to vascular plants has grouped the Dof proteins analyzed into seven subfamilies (Moreno-Risueño *et al.*, 2007). According to this study, and the data presented here, the Dof5 protein from maritime pine would be in subfamily B that possibly originated before the divergence of the angiosperm and the gymnosperm ancestors (Moreno-Risueño *et al.*, 2007). Considering that, as reported for other protein families (Pinyopich *et al.*, 2003), the establishment of new gene functions could occur by duplications events and subsequent diversification, *PpDof5* should be one of the most ancient *Dof* genes in pine suggesting it is involved in essential and ancient functions concerning the tree. Consistently, the recent analysis of *Dof* gene families in poplar, Arabidopsis and rice has suggested that multiple modes of gene evolution are involved in gene diversification after duplication (Yang *et al.*, 2007).

PpDof5 has been localized in the nucleus of onion epidermal cells, indicating that the protein contains a putative nuclear location signal that could be involved in the targeting to the nucleus. Some conserved motifs along the Dof sequences in different species suggest a common origin. Furthermore, some of these motifs are in close proximity to the Dof domain (Moreno-Risueño *et al.*, 2007) and bear a nuclear localization signal (NLS). In PpDof5 the putative NLS adjacent to the Dof domain could account for the nuclear localization of GFP fusion product.

Pine Dof5 transcripts, although ubiquitously expressed, are particularly abundant in non-photosynthetic tissues and are almost absent in needles. This pattern of expression suggests a predominant role for PpDof5 in non-photosynthetic tissues. Analysis of the level of expression of PpDof5 during development in pine suggests its involvement in a regulatory network essential to development. Thus, the transcription factor is expressed during germination when organ development takes place and there is a high demand for nitrogen compounds. We have previously shown that GS1b plays a key role in the mobilization of seed reserves during early stages, providing glutamine for growth and development. The expression of the *GS1b* gene is associated with the vascular system of roots, stems and cotyledons (Avila *et al.*, 2001a). Furthermore, *GS1b* transcripts are exclusively located in the procambial cells of developing pine embryos prior to the differentiation of mature vascular elements (Pérez-Rodríguez *et al.*, 2006).

Gel shift assays (Figure 5) reveal the interaction *in vitro* between PpDof5 and the *GS1b* promoter and support the ability of PpDof5 to activate transcription of *GS1b* in both tobacco leaves and pine protoplasts (Figures 4 and 6). Moreover, functional deletion analysis (Figure 7) has shown that the regulatory effect of Dof 5 is suppressed when truncated forms of the promoter without the Dof-binding element are used. That allows us to speculate about the role of PpDof5 in controlling the presence of GS1b protein in the vascular system in order to account for the reassimilation of ammonium liberated from metabolic processes and the generation of glutamine for nitrogen transport. Thus, PpDof5 accounts for an essential and efficient mechanism for regulating the level of GS1b, a key enzyme in reassimilation of the nitrogen released in several metabolic reactions such as phenylalanine deamination or glycine decarboxylation which are quantitatively very important during lignification in trees (Cantón *et al.*, 2005; Rajinikanth *et al.*, 2007).

The presence of canonical binding core sequences in the *GS1a* promoter led us to determine whether PpDof5 could have a relevant role as a regulator of *GS1a* transcription. Our results indicate that, as occurs with the *GS1b* promoter, PpDof5 is able to bind a region of the *GS1a* promoter *in vitro* (Figure 5) and to regulate gene transcription *in vivo*, either in tobacco leaves or pine protoplasts (Figures 4 and 6). A regulatory region has been identified by functional deletion analysis (Figure 7). The antagonist function related to the *GS1b* promoter immediately suggests a mechanism for the transcriptional control of *GS1* gene expression in non-photosynthetic and photosynthetic organs of pine. As shown in Figure 8, in stems and roots, where GS1a is faintly detected, *PpDof5* is highly expressed. This is in agreement with its proposed role as a suppressor of *GS1a* expression. In contrast, the accumulation of PpDof5 would activate transcription of the *GS1b* gene in the same organs, where the GS1b enzyme has the role of generating glutamine for nitrogen transport and recycling the ammonium released in lignin biosynthesis. Transcriptional regulation mediated by PpDof5 could also explain, at least in part, the expression of *GS1a* and *GS1b* genes in photosynthetic organs of pine. Thus, a low

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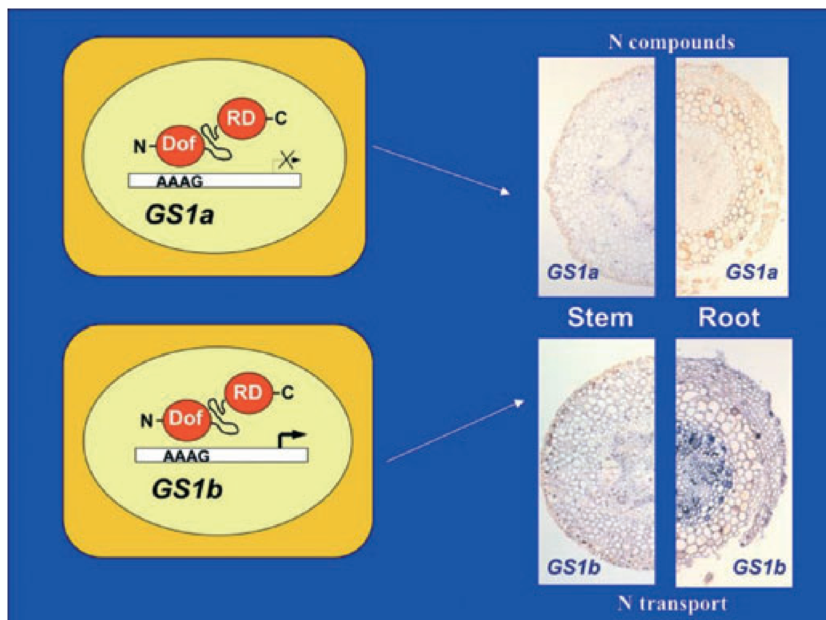

Figure 8. Proposed model of how the PpDof5 transcription factor regulates pine GS gene expression.
The schematic representation is based on the experimental data reported here and in a previous work by Avila *et al.* (2001a). Dof, DNA-binding domain; RD, transcriptional regulatory domain. The interaction of other transcription factors cannot be ruled out because the Dof domain mediates both DNA binding and protein–protein interactions (Yanagisawa, 2004). The main functional role of pine GS1 proteins is also indicated.

level of the transcription factor in pine needles would turn *GS1b* expression off whereas *GS1a* expression would be activated by lack of the transcriptional suppressor. The GS1a isoform has a crucial role in the biosynthesis of nitrogen compounds in photosynthetic tissues.

A question arises from the results reported here: How can the reciprocal regulation of *GS1* genes be explained? The first consideration to answer the question is structural. Even though the target AAAG core sequences are the same for either gene, their entire regulatory regions are largely unrelated (Gómez-Maldonado *et al.*, 2004b). Furthermore, the oligonucleotide sequences (*GS1a* and *GS1b*) used for the gel shift assays contain non-coincident *cis*-elements in the sequences flanking the Dof binding sites (Figure S2 in Supplementary Material). Since the accumulation of a given transcript is the result of a complex combination of regulatory processes, different elements in the surroundings of the canonical Dof binding sites could explain, at least in part, the differential regulation observed. These results, in addition to other structural and functional differences (Gómez-Maldonado *et al.*, 2004a,b,c), indicate that unique regulatory regions in each promoter are controlling the transcriptional activity of *GS* genes and, hence, are responsible for the distinct expression patterns (Avila *et al.*, 2001a). Nevertheless, further research work is needed to define the molecular mechanisms that are involved.

In conclusion, the current study provides a new clue about how one component of the transcriptional machinery may control the spatial distribution of two different isoenzymes of a key process in ammonium assimilation for glutamine biosynthesis. Furthermore, PpDof5 possibly originated before the divergence of angiosperms and gymnosperms and, therefore, the transcriptional control of *GS* genes, as reported here, probably reflects an ancient regulatory mechanism in plants.

Conifer *GS1a* and *GS1b* genes have been suggested to be orthologs of genes encoding chloroplastic and cytosolic GS isoforms in angiosperms (Avila-Sáez *et al.*, 2000). We have found that the promoter regions of the genes for GS1 and GS2 in angiosperms contain Dof-binding sites; whether or not Dof factors might control the differential expression of *GS* genes in angiosperms remains to be determined.

Experimental procedures

Plant material

Maritime pine (*P. pinaster* Ait.) seeds used in all experiments were provided by 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 pine PpDof5 cDNA clone and sequence analysis

A λ-ZAP composite cDNA library constructed from developing xylem was used for mass sequencing and EST generation (Cantón *et al.*, 2003). Two clones containing Dof sequences were selected and the cDNA inserts analyzed by agarose gel electrophoresis (data not shown). One of both ESTs, named PP100A03, harboured an insert of 1.7 kb that was sequenced by standard procedures using and ABI automated sequencer (Applied Biosystems, http://www.appliedbiosystems.com/). Sequence alignments and phylogenetic analyses were performed using the ClustalW program (Thompson *et al.*, 1994).

RT-PCR analysis

A RT-PCR analysis was performed to study variation in the level of transcription of the genes *GS1a*, *GS1b* and *PpDof5* at different

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stages during pine development. Total RNA for RT-PCR was isolated from three embryo stages: dry embryos (DE), embedded embryos (EE) and germinating embryos (GE). Cotyledons, shoots and roots were taken from pine seedlings (seedlings with a cotyledon length of 2.5 cm) and from 1-year-old trees. Total RNA was isolated and purified from frozen tissue using the Aurum Total RNA kit (Bio-Rad, http://www.bio-rad.com/). First-strand cDNA synthesis was primed with oligodT and catalyzed by AMV Reverse Transcriptase according to the manufacturer's instructions (Roche, http://www.roche.com/). The primers used for PCR amplification were as follows: *GS1a* forward 5′-TAAATTACAAAGTGGAG-3′, reverse 5′-CACAA-AGAATATATCAT-3′; *GS1b* forward 5′-AGTGAGAGTAACATTGA-3′, reverse 5′-CGCAAGCAAGCAATAAC-3′; *PpDof5* forward 5′-GAT-AACAACCTACAGGT-3′, reverse 5′-TTCCACACAGACAGTAA-3′. As a control, a fragment of 300 bp of the constitutive *eiF4a-2* was amplified by using the following primers: 5′-GGCTTCAAGGATCA-GAT-3′ and 5′-CTGGTCCATGTCTCCATG-3′. The resulting PCR products were separated by agarose gel electrophoresis, transferred to nylon membranes and hybridized with specific ^{32}P-labelled probes. Quantification was performed using the bio-imaging analyzer BAS-1500 (Fuji Photofilm, http://www.fujifilm.com/).

Epidermal onion cell transformation

To create the 35S::Dof5-GFP construct for particle bombardment of onion cells, an in-frame translational fusion of the *PpDof5* ORF with the GFP reporter gene was undertaken in the plasmid pCAMBIA 1302 containing the *CaMV 35S* promoter. As a control we used the plasmid without the Dof5 insert. Inner epidermal layers of onion bulbs (*Allium cepa*), purchased locally, were peeled and placed onto half-strength MS agar medium as described by Borrell *et al.* (2002). Particle bombardment was carried out with a biolistic helium gun device PDS-1000/He apparatus from Bio-Rad. Each shot delivered 120 ng of DNA using a rupture disc of 900 p.s.i. at a distance between the macrocarrier and the sample of 9 cm. After 24 h of incubation at 22°C in the dark the fluorescence emission was observed. As a control for nuclei location, tissue samples were stained with DAPI. All observations were made with a Nikon E 800 microscope (http://www.nikon.com/).

Electrophoretic mobility shift assays

The PpDof5 protein was expressed in *E. coli* BL21-codonPlus-(DE3)-RIL (Stratagene) by cloning its ORF into the *Xho*I restriction site of pTrcHisB (Invitrogen, http://www.invitrogen.com/). Recombinant protein was induced with 1 mM isopropyl-β-D-thio-galacto-pyranoside (IPTG) for 3 h at 25°C. Following induction of protein expression in the cells, the N-terminus 6× His-tagged protein was purified by affinity chromatography using an Ni-NTA column (Qiagen, http://www.qiagen.com/) under native conditions. The oligonucleotide probes, described in Figures 5 and 6, respectively, were generated by annealing complementary oligonucleotides designed to create 5′ overhangs that were end-filled with Klenow DNA polymerase in the presence of [α^{32}P]dCTP. Each binding mix contained in 20 μl of solution 2 μl of 10× binding buffer: 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) pH 7.6, 500 mM NaCl, 10 mM DTT, 10 mM EDTA, 50% glycerol, 2 μg salmon sperm DNA and 0.5 μg of poly d(I+C) as an unspecific competitor. This binding solution was incubated with the radiolabelled probe (40 000 c.p.m. reaction^{-1}) for 30 min. At the end of the incubation period 1/10th of the mix volume of loading buffer was added. The DNA–protein complexes were analyzed by electrophoresis on 6% (w/v) polyacrylamide, 2% glycerol gels run in 0.25× TRIS-borate-EDTA (TBE) buffer at 130 V for 2 h at 4°C. Gels were dried under vacuum and autoradiographed using film (X-OMAT S; Kodak, http://www.kodak.com/).

Transient expression analysis by agroinfiltration of tobacco leaves

Binary constructs for tobacco transformation were performed as follows. The promoterless::GUS (pBI101) plasmid was used to introduce *GS1a* and *GS1b* gene promoters and these were the reporter constructs. Two other constructs, *CaMV 35S* promoter::GUS (pBI121) and promoterless::GUS (pBI101), were used as positive and negative controls, respectively. The effector construct containing *PpDof5* was performed in the pCAMBIA 1302 vector.

Co-transfection experiments were performed essentially according to the method of Yang *et al.* (2000). *Agrobacterium tumefaciens* strains EHA 105 containing either a binary effector plasmid or a reporter construct, were co-infiltrated into nearly fully expanded leaves of 6- to 7-week-old tobacco (*N. benthamiana*) plants, using a 1-ml syringe. After agroinfiltration, tobacco plants were maintained in a growth chamber at 22°C, 16 h light for 2 days.

Histochemical localization of β-glucuronidase activity was performed as described by Jefferson (1987) using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc; Clontech, http://www.Clontech.com/).

Transient expression assays in pine protoplasts

In order to construct fusions between the *GS1a* and *GS1b* promoters and the GUS reporter gene for transient expression assays in pine protoplasts, both promoters and their deletions were inserted into the polylinker of the GUS-encoding plasmid pBI221 (Jefferson *et al.*, 1987) in frame with the GUS gene by replacing the full *CaMV 35S* promoter. Pine protoplasts were prepared essentially as described elsewhere (Gómez-Maldonado *et al.*, 2001). For electroporation, 50 μg of the following DNAs were mixed: either *GS1a* or *GS1b* promoter:pBI221 and Dof5:pJIT60. Electroporation conditions and GUS analysis was as described before (Gómez-Maldonado *et al.*, 2004c).

Analysis of GUS expression

Fluorogenic analysis of GUS reporter expression was accomplished according to standard methods (Jefferson *et al.*, 1987) with minor modifications. The reactions were carried out at 37°C. The resulting fluorescence was measured at 30-min intervals using a FL6000 Bio-TER Fluorescence Reader using methylumbelliferone (MU) as a standard.

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Supporting Information

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Figure S1. Phylogenetic analysis of full-length protein sequences of Dof genes in poplar, Arabidopsis and rice.
Figure S2. PLACE signal scan of the oligo sequences used in the binding assays.
Table S1. The Dof binding site presents in the GS promoters of Arabidopsis, poplar and pine.
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