

Characterization and developmental expression of a glutamate decarboxylase from maritime pine

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Abstract Glutamate decarboxylase (GAD, EC 4.1.1.15) is a key enzyme in the synthesis of γ -aminobutyric acid (GABA) in higher plants. A complete cDNA encoding glutamate decarboxylase (GAD, EC 4.1.1.15) was characterized from *Pinus pinaster* Ait, and its expression pattern was studied to gain insight into the role of GAD in the differentiation of the vascular system. Pine GAD contained a C-terminal region with conserved residues and a predicted secondary structure similar to the calmodulin (CaM)-binding domains of angiosperm GADs. The enzyme was able to bind to a bovine CaM-agarose column and GAD activity was higher at acidic pH, suggesting that the pine GAD can be regulated *in vivo* by Ca^{2+} /CaM and pH. A polyclonal antiserum was prepared against the pine protein. GAD expression was studied at activity, protein, and mRNA

level and was compared with the expression of other genes during the differentiation of the hypocotyl and induction of reaction wood. In seedling organs, GABA levels closely matched GAD expression, with high levels in the root and during lignification of the hypocotyl. GAD expression was also induced in response to the production of compression wood and its expression matched the pattern of other genes involved in ethylene and 2-oxoglutarate synthesis. The results suggest of a role of GAD in hypocotyl and stem development in pine.

Keywords Glutamate decarboxylase · GABA · Isocitrate dehydrogenase · Pine · Reaction wood · Vascular development

Abbreviations

| | |
|------------------------|--|
| ACO | 1-Aminocyclopropane-1-carboxylic acid oxidase |
| ACS | 1-Aminocyclopropane-1-carboxylic acid synthase |
| APX | Ascorbate peroxidase |
| AOX | Ascorbate oxidase |
| CaM | Calmodulin |
| GAD | Glutamate decarboxylase |
| LAC | Laccase |
| NADP ⁺ -IDH | NADP ⁺ -isocitrate dehydrogenase |

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Introduction

γ -Aminobutyric acid (GABA) is a non-proteinogenic amino acid with well-established functions as neurotransmitter in animals (Bormann 2000). In plants, the production of GABA has extensively been reported during both biotic and abiotic stresses, including pathogen infection, organ

damage, temperature shock, and hypoxia (reviewed in Bouche and Fromm 2004). Peaks in GABA levels have also been detected in other physiological processes, such as in senescence (Masclaux et al. 2000) or fruit ripening (Gallardo et al. 1993; Cercós et al. 2006). Currently, GABA synthesis is thought to make a contribution to different aspects of plant biology, including regulation of C/N balance, buffering of cytosolic pH, protection against insects and oxidative stress, and osmoregulation (reviewed in Bouche and Fromm 2004). In addition, GABA has been also proposed to be a signaling molecule in plants (Bouche and Fromm 2004). Plant cells can produce GABA by a reaction catalyzed by cytosolic glutamate decarboxylase (GAD, EC 4.1.1.15), by reversible mitochondrial aminotransferase reactions, and as a by-product associated with putrescine degradation (Fait et al. 2008). From these possible sources, GABA synthesis by the reaction catalyzed by GAD is considered to be the most important in plant tissues (Bouche and Fromm 2004). In fact, GABA aminotransferases are thought to be involved in the recycling of GABA levels by the mitochondrial GABA-shunt pathway (Bouche and Fromm 2004; Fait et al. 2008). However, very limited information is currently available about the contribution of putrescine degradation to GABA levels in plants (Cona et al. 2006).

GAD has been studied in only a small number of herbaceous plants. GAD activity results in the decarboxylation of glutamate to GABA in a proton-consuming reaction that requires pyridoxal-5'-phosphate as a cofactor. The protein is encoded by a small family of nuclear genes in angiosperms (Akama et al. 2001). According to the information in databases, the polypeptide subunit size of plant GAD is 55–58 kD. However, the molecular features of the native enzyme are not clear, and holoenzymes of 550 and 340 kD have been reported (Zik et al. 2006). All plant GAD sequences in GenBank share a well-conserved central domain that is involved in the interaction with the cofactor pyridoxal-5'-phosphate, and most of the sequences also contain a calmodulin (CaM)-binding domain in the carboxyl terminal of the protein. In vitro GAD activity is increased by acidic pH and by Ca²⁺-CaM binding (Crawford et al. 1994; Arazi et al. 1995; Bouche and Fromm 2004), indicating that the production of GABA in response to different types of stress is intimately associated with activation of the GAD enzyme. The regulation of GAD by CaM strongly suggests that GABA production could be involved in signaling pathways. In *Arabidopsis*, it has been reported that GABA levels function as a signal that controls pollen tube growth (Palanivelu et al. 2003), and in tobacco and rice, it was found that GABA levels affect cellular elongation in the stem in vivo (Baum et al. 1996; Akama and Takaiwa 2007). Although no molecular receptor for GABA has been characterized in plants so far, the regulation of a

large set of 14-3-3 proteins by GABA was recently reported in *Arabidopsis* (Lancien and Roberts 2006), and the existence of binding sites for GABA in pollen protoplasts has been reported in tobacco (Yu et al. 2006). These data strongly suggest a role for GABA as a signaling molecule in plants. In support of a role in signaling, GABA can be transported through phloem (Beuve et al. 2004), and GABA production has been linked to ethylene synthesis and stem development in seedlings (Kathiresan et al. 1997, 1998).

Studies about GABA biosynthesis and the functional role of the metabolite in trees are very limited. According to EST data from pine and poplar, GAD expression has been detected in the cambial zone (Cantón et al. 2004; Sterky et al. 2004). To study the role of GABA in tree development, we characterized a GAD from *Pinus pinaster* and its expression was compared with other genes involved in 2-oxoglutarate and ethylene synthesis during differentiation of the secondary vascular development in seedlings.

Materials and methods

Plant material and growth conditions

Pinus pinaster Ait. seeds from Sierra Bermeja (Málaga, Spain) were provided by the Consejería de Medio Ambiente (Junta de Andalucía). Seeds were imbibed in deionized water for 24 h under continuous aeration, and germinated and grown as previously described (Cánovas 1991). No external nitrogen was added to the plants. In the studies with 7-month old seedlings, the plants were grown in 1.5 L of COMPO SANA substrate (COMPO Agricultura S.L., Barcelona, Spain), which was refreshed every 15 days by replacing the top 200 ml with new substrate.

Sequence analysis and secondary structure prediction

Random sequencing of a cDNA library from differentiating xylem of *P. pinaster* resulted in the initial identification of two cDNA clones with sequences similar to GADs from angiosperms (Cantón et al. 2004). Characterization of these clones indicated that they corresponded to the same gene product, and the larger one (1875 nt) was fully characterized by subcloning and sequencing. This clone contained a full-length cDNA encoding GAD, and the sequence was deposited into GenBank as DQ 125946. Further analysis of the same library identified five additional partial cDNAs deposited in Genbank that correspond to the same gene product. Sequence alignments and phylogenetic analyses were performed with the Clustal X 1.83 program (Thompson et al. 1997), and the unrooted tree was viewed using Tree

View 1.6.6. (Page 1996). The NCBI accession number of the sequences considered were as follow: Ath, *Arabidopsis thaliana* (Ath1, NM 121739; Ath2, AF 428294; Ath3, NM 126261; Ath4, NM 126262; Ath5, NM 112657); Bju, *Brasica juncea* (Bju1, AY 559319 and AY 559320; Bju3, AY 559318); Csi, *Citrus sinensis* DQ001727; Gmax, *Glycine max* AB240965; Hvu, *Hordeum vulgare* AF 521177; Les, *Lycopersicon esculentum* (Les1, X80840; Les2, AB359914; Les3, AB359915); Nta, *Nicotiana tabacum* (Nta1, AF 020425, AF 020424, AF 352732, AF 506366 and U54774; Nta6, AF353615); Osa, *Oryza sativa* (Osa1, NM 001068533; Osa2, AB 056061; Osa3, NM 001056025; Osa4, AF377946; Osa5, AP006461; Osa6, NM 001059454); Pet, *Petunia x hybrida* L 16797; Ppa, *Physcomitrella patens subsp. patens* (Ppa1, XM 001764442; Ppa2, XM 001758870; Ppa3, XM 001753263); Ppi, *P. pinaster* DQ 125946; Ptrs, *Populus tremula* sequence provided by Dr. F. Sterky (Royal Institute of Technology, Stockholm), *P. tremula x P. alba* (PtrPal) DQ 125945, and *P. trichocarpa*, Ptri4, XM 002305891; Ptri, *P. trichocarpa* (Ptri1, coding sequence deduced from GAD gene in LGXII (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html); Ptri2, XM 002312455; Ptri3, XM 002314713; Ptri5, XM 002327560; Ptri6, XM 002305890); and Vvi, *Vitis vinifera* (Vvi1, CU459222; Vvi2, AM460203). The prediction of secondary structure was made with the nnpredict program (Kneller et al. 1990).

Nucleic acid isolation and analysis

Genomic DNA was prepared according to Dellaporta et al. (1983). Total RNA was isolated following the procedure described by Chang et al. (1993). DNA digestion and Southern and Northern blotting were carried out following standard procedures (Ausubel et al. 2009). For Northern blots of GAD expression, a probe corresponding to the 3' non-translated sequence of pine GAD cDNA was used for hybridization. Prehybridizations and hybridizations were performed at 65°C. After hybridization, membranes were washed at 65°C in 0.1 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7) and 0.1% SDS for Northern blots, and at 65°C in 0.5 × SSC, 0.1% SDS for Southern blots before exposure. In the expression studies of other genes, probes were prepared from homologous cDNAs corresponding to the following accession numbers: NADP⁺-isocitrate dehydrogenase (NADP⁺-IDH), AY344584; cytosolic ascorbate peroxidase (APX), BX251255; ascorbate oxidase (AOX), BX255089; laccase (LAC), BX249680; and ACC oxidase (ACO), BX784199. In the case of ACC synthase (ACS), a partial cDNA sequence of 344 bp was cloned by RT-PCR using primers corresponding to conserved regions: 5'CTGAGATGGAGAAGCTGGCG3' and 5'TCTGAAGCCTGGAAGGCC3'.

Purification of recombinant pine GAD from *Escherichia coli* and production of a polyclonal antiserum

The full-length cDNA of *P. pinaster* GAD containing the open reading frame sequence (PpGAD) was amplified by PCR with a 5' primer with an overhang region containing a unique restriction site for NdeI, an enterokinase cleavage site and a six-histidine tag preceded by a universal ATG start codon (5'-CGCCATATGCACCATCATCATCATCATGA CGACGACGACAAGATGGCTCTGAGCAGCGC-3'), and a 3' primer with an overhang region containing an EcoRI restriction site (5'-CGGAATTCAGCCATTTGAATGG GGC-3'). The PCR reaction was carried out with Turbo Pfu polymerase (Stratagene, Cedar Creek, TX, USA), and the amplified products were digested with NdeI and EcoRI and subcloned into the expression vector PET30a. The resulting construct was digested with HindIII and re-ligated to create a new one for the expression of a truncated GAD version lacking 93 residues from the C-terminal end, a region that includes the putative CaM-binding domain (Δ GAD). The *E. coli* strain BL21-codonPlus-(DE3)-RIL (Stratagene) was transformed with both plasmids separately. For the expression of the recombinant proteins and purification of recombinant GAD, cells were grown at 37°C with shaking in Luria-Bertani broth containing 15 μ g ml⁻¹ kanamycin and 34 μ g ml⁻¹ chloramphenicol until a cell density (OD₆₀₀) of 0.6–0.8 was reached. Isopropyl- β -D-thiogalactoside (IPTG) was then added at a final concentration of 1 mM, and incubation was continued for 2 h with shaking at 37°C. Cells were collected by centrifugation at 4,000g and resuspended in a buffer containing 100 mM NaH₂PO₄, 10 mM Tris-Cl, and 6 M guanidine hydrochloride at pH 8.0. Cell debris was removed by centrifugation at 10,000g, and the soluble protein fraction in the supernatant was fractionated by SDS-PAGE for analysis. For the purification of GAD, the soluble fraction was fractionated through an affinity chromatography Ni-NTA column (Qiagen, Crawley, Sussex, UK). For CaM-binding studies, protein preparations were fractionated through an agarose-calmodulin column following the manufacturer's instructions (Sigma, Ref P2277). The bound proteins were eluted with 1–2 mM EDTA.

Antibodies against pine GAD were raised in rabbits by immunization with the recombinant protein as described by Palomo et al. (1998). Several dilutions of the serum were tested against pine GAD in crude extracts, and a 1:20,000 dilution was selected to detect GAD polypeptide in Western blots.

Protein extraction, SDS-PAGE, and western-blot analysis

Total soluble proteins were extracted as described previously (Gallardo et al. 1999). Plant tissues were frozen with

liquid nitrogen and ground into a powder as an initial step for extraction. Protein content was estimated by the Bradford method (Bradford 1976) using bovine serum albumin as a standard. Protein profiles were analyzed by fractionation of samples by SDS–PAGE followed by Coomassie blue staining. Specific detection of proteins was conducted by Western blot analysis using antisera raised against pine NADP⁺-IDH (Palomo et al. 1998) or recombinant GAD.

GAD activity and determination of GABA levels

Glutamate decarboxylase activity and GABA levels were determined spectrophotometrically at 340 nm using the enzymatic GABASE assay (Sigma–Aldrich) as described by Zhang and Bown (1997) with minor modifications. Plant material was ground in liquid nitrogen and ground tissue (0.5 g) was extracted at 4°C in 150 mM potassium phosphate, 5 mM EDTA, 1 mM magnesium chloride, 0.5% (w/v) polyvinylpyrrolidone, 3 mM 2-mercaptoethanol, 0.2 mM pyridoxal-5'-phosphate, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, pH 5.8 or 7.0. The reaction mixture (1 ml) for determination of GAD activity contained 150 mM potassium phosphate, 10 mM sodium chloride, 0.2 mM pyridoxal-5'-phosphate, and 50 mM glutamate and was adjusted to pH 5.8 or 7.0. For the determination of GAD activity in the presence of calmodulin (CaM), 500 µM Ca²⁺ and 200 nM bovine CaM were added into the reaction mix. After 10 min at 30°C, the reaction mix were boiled for 5 min and 50 µl was taken for GABA detection using the GABASE assay. Blanks without incubation and with previous inactivation of enzymes by boiling for 5 min were considered in the determinations of GAD activity. For the determination of GABA in tissue samples, extracts from frozen homogenate were immediately boiled for 5 min for the inactivation of the enzymes, and absorbance was monitored before and after the addition of the commercial enzymatic preparation and 2-oxoglutarate in the reaction mix.

Water, cell wall and lignin content, and histochemical analysis of pine hypocotyl

Determination of water content and cell wall and lignin (Klason method) was carried out as described by Jing et al. (2004). For histochemical analysis by optical microscopy, pine hypocotyl was fixed in 1.25% glutaraldehyde in 100% methanol by the freeze substitution method (Regan et al. 1999). Samples were embedded in paraffin, and 10-µm-thick sections were prepared as described previously (Pascual et al. 2008). Staining with hematoxylin and eosin was carried out following standard procedures (Charboneau et al. 2004) with minor modifications. Gill's hematoxylin and eosin Y solutions were used for 3 min and 20 s, respec-

Fig. 1 Characteristics of the GAD protein sequence from *Pinus pinaster* and phylogenetic analysis of plant GADs. **a** Alignment of pine GAD sequence (Ppi) with other plant GADs. Identical and similar residues found in the consensus GAD sequence from angiosperms (Angiosp) are shown in *black* or *gray*, respectively. The consensus GAD sequence was obtained after alignment of angiosperm sequences with putative CaM-binding domains (Suppl. Fig. S1). Residues in the consensus angiosperm sequence indicated by an X correspond to positions in which four or more different residues are found in different species. A dash represents a gap introduced to optimize the alignment. Other sequences included are GAD1 from *Oryza sativa* (Osa1), which has a typical CaM-binding domain, and *O. sativa* GAD2 (Osa2) and a Bryophyta GAD sequence (Ppa2), which lack the CaM-binding domain. The pyridoxal-5'-phosphate and catalytic domain are underlined with a continuous and a dashed line, respectively. The CaM-binding domain is *boxed*. Relevant residues involved in catalysis and in the interaction with CaM are marked with *asterisks*. **b** Secondary structure and alpha helix wheel-model prediction for the CaM-binding domain of pine and rice GAD sequences. The prediction of Osa1 and pine sequences correspond to an amphipathic alpha helix with most of the charged residues located in one half. H, residue in a predicted alpha helix conformation; E, residue in a predicted beta-strand conformation; **c** Southern blot of pine genomic DNA hybridized with P³²-pine GAD cDNA. The multiple bands indicate the existence of several genes that encode GAD in the *Pinus pinaster* genome. **d** Phylogenetic analysis of plant GAD sequences. Consistent bootstrap groups are indicated with bootstrap values. Osa2 and Bryophyta GAD sequences (ppa1 and pp2) lack the CaM-binding domain

tively; sections were then washed twice with 95% ethanol and one time with 100% ethanol.

Results

Molecular characterization of pine glutamate decarboxylase

The sequencing of a *Pinus pinaster* cDNA library from differentiating xylem (Cantón et al. 2004) allowed the identification of several clones similar to angiosperms GAD. Full characterization of the cDNA inserts indicated that all cDNAs corresponded to the same mRNA and allowed the identification of a 1875-nt full-length cDNA (DQ 125946) with an identity index of 64.1–67.8% with angiosperms GAD DNA sequences (data not shown). The derived primary structure of the complete cDNA corresponded to a polypeptide of 509 residues with a molecular size of 57.2 kD, an estimated pI value of 5.74, and a similarity index of 69.9–76.6% with amino acid sequences of angiosperm GADs (not shown). The highest homology to plant GADs is found in the central part of the polypeptide, corresponding to the catalytic site and the pyridoxal-5'-phosphate-binding domain, and all relevant residues involved in catalysis were conserved (Fig. 1a). The C-terminal region of pine GAD shows a 28-residue region with features similar to a CaM-binding domain, including one flanking lysine at each edge and a tryptophan residue (W497) in the WKK

a

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Ppi 1 MVLST----SATDSGSIHSTFASRYVDESLPRFQVPRFSTPKAAAYQIIDEMLMDGNPRLNLSFVTTWMEPECDKLMDSINKNYVDMDEYFVTTTELQNRVNIATA
Osa1 1 MVLSTVAHSDTAQPVQVSHIFASRYVDESLPRFQVPRFSTPKAAAYQIIDEMLMDGNPRLNLSFVTTWMEPECDKLMDSINKNYVDMDEYFVTTTELQNRVNIATA
Angiosp 1 MVLSTIASSDVSV---HSTFASRYVDESLPRFQVPRFSTPKAAAYQIIDEMLMDGNPRLNLSFVTTWMEPECDKLMDSINKNYVDMDEYFVTTTELQNRVNIATA
Osa2 1 MVLHVEAVEDESE---AAAAVFASRYVDDPVPRENGERSISKAAAYQIIDEMLMDGNPRLNLSFVTTWMEPECDKLMDSINKNYVDMDEYFVTTTELQNRVNIATA
Ppa2 1 MVLSECKSMKRTKGHTDIDMVFASRYVAQSLPRFQVPRFSTPKAAAYQIIDEMLMDGNPRLNLSFVTTWMEPECDKLMDSINKNYVDMDEYFVTTTELQNRVNIATA

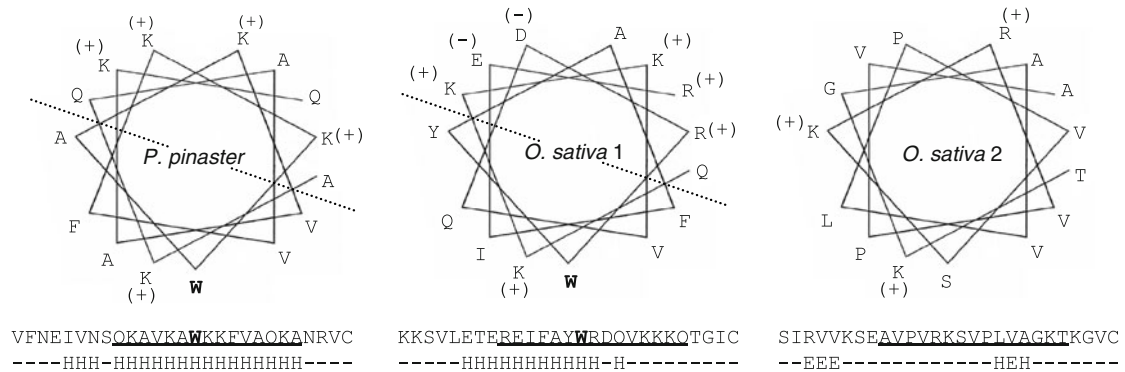
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Osa2 107 NLFNAPLGDDEEAVGVGTVGSSEAIMLAGLAFKRRKQWQNRKAEGKPYDKPNIVTGANVQVCWEKFARYFEVELKEVKIKKDYIIMDPKAVEMVDENTICVAAILGSTY
Ppa2 109 NLFNAPLGDDEEAVGVGTVGSSEAIMLAGLAFKRRKQWQNRKAEGKPYDKPNIVTGANVQVCWEKFARYFEVELKEVKIKKDYIIMDPKAVEMVDENTICVAAILGSTY

Ppi 213 NGEFEDVKLINDLLLEKKNKETGWDTPIHVDAASGGFIAPFLYPELEWDFRPLPVKSIIVSGHKYGLVYAGIGWVIWRKEDLPEELIFHINYLADQPTFTLNFSGKAS
Osa1 218 NGEFEDVKLINDLLLEKKNKETGWDTPIHVDAASGGFIAPFLYPELEWDFRPLPVKSIIVSGHKYGLVYAGIGWVIWRKEDLPEELIFHINYLADQPTFTLNFSGKSS
Angiosp 213 NGEFEDVKLINDLLLEKKNKETGWDTPIHVDAASGGFIAPFLYPELEWDFRPLPVKSIIVSGHKYGLVYAGIGWVIWRKEDLPEELIFHINYLADQPTFTLNFSGKSS
Osa2 216 NGEFEDVKLINDLLLEKKNKETGWDTPIHVDAASGGFIAPFLYPELEWDFRPLPVKSIIVSGHKYGLVYAGIGWVIWRKEDLPEELIFHINYLADQPTFTLNFSGKSS
Ppa2 218 NGEFEDVKLINDLLLEKKNKETGWDTPIHVDAASGGFIAPFLYPELEWDFRPLPVKSIIVSGHKYGLVYAGIGWVIWRKEDLPEELIFHINYLADQPTFTLNFSGKAS

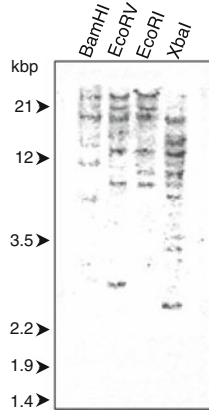
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Angiosp 322 QVIAQYQIIRLGFEGYKNMNCNENALIREGLEKTRGFWISKAGVPLVAFSLKDSRYEVEIISEHLRRFGWIVPAYTMAPDAQHVTILRVVIREDFRRLAER
Osa2 325 QIAQYQIIRLGFEGYKSMNCOMESARTIREGLEKTRGFWISKAGVPLVAFSLKDSRYEVEIISEHLRRFGWIVPAYTMAPDAQHVTILRVVIREDFRRLAER
Ppa2 327 QVIAQYQIIRLGFEGYKSMNCOMENAKITLITGRENIGREKISKAGVPLVAFSLKDSRYEVEIISEHLRRFGWIVPAYTMAPDAQHVTILRVVIREDFRRLAER

Ppi 431 LVVDIEKVLHELDITLPSAREIVASLVGDHPELKEVKDLEID-VTCERK-SAVFNEIVNSQKAVKAKKFEVAQ-KAN--RVC 509
Osa1 436 LVSDIEKVLHELDIAHSAQVLKISSRIAKQ-----QSDDGVVTKKSVLEPEREIFA-----YMDGQV--KKKQTGIC 501
Angiosp 431 LVVDIEKVLHELDITLPAVAKKTLAXX-----XNEXX--XKKKEXEKREITFX-----XWKEVXX-KKXXXXVC 496
Osa2 434 FISHRMALDEHDLAARAPVPRVQITIELG-----PARTAGEEA-----SIRVVKSEAVP-----V-KKSVPLVAGTKGVC 500
Ppa2 436 LVVDIEKVLHELDHAREPRLIEVITARVAQENREANLDLPTTPDA-V--K-ATAAFNDFVVEGHHGKKRQOHGHGEGHHGHPATTATATATTARNPPFTR 533
    
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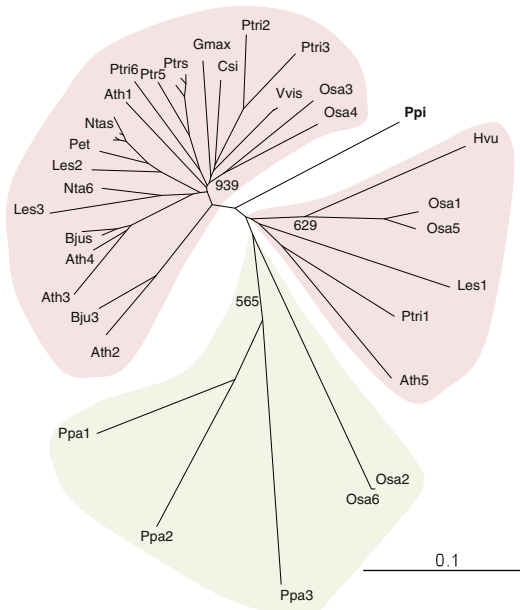
b



c



d



sequence that is typically conserved in GADs that bind CaM (Baum et al. 1993; Arazi et al. 1995; see box in Fig. 1a). The alignment of GAD sequences also indicated that the CaM-binding features of pine sequence are absent in two GAD sequences reported in rice (Osa2 in the alignment of Fig. 1a) that do not bind CaM in vitro (Akama et al. 2001) and in all full-length sequences from Bryophyta available to date (*Physcomitrella*, Ppa2 in Fig. 1a). It is also interesting to note that the putative CaM-binding domain in the pine sequence contains a central—SQKAVK—sequence that is absent in other plant GADs (Fig. 1a, Suppl. Fig. S1). To determine if this sequence could alter the predicted secondary structure of a typical CaM-binding domain (O’Neil and DeGrado 1990), we compared the predicted structure of the residues flanking W497 in pine GAD with rice GAD sequences, which differ in their capacity to bind CaM in vitro. The predicted secondary structures and wheel alpha helix representations are shown in Fig. 1b. Residues flanking the conserved tryptophan residue corresponded to an alpha helix in the pine sequence and in the Osa1 sequence, of which the GAD product has been reported to bind CaM in vitro (Akama et al. 2001; Fig. 1b). The wheel representation for pine and Osa1 proteins also predicts an amphipathic alpha helix, in which the conserved tryptophan is located in the hydrophobic part and most charged residues are inside the hydrophilic half (Fig. 1b). These features, a predicted alpha helix and amphipathic distribution of residues, correspond to the features of CaM-binding domains (O’Neil and DeGrado 1990). In contrast to pine and Osa1 proteins, no alpha helix was predicted for GAD2 from *O. sativa* (Fig. 1b), which lacks the conserved tryptophan residue and does not bind CaM in vitro (Akama et al. 2001). These data suggest that pine GAD has similar features in the C-terminal region to other angiosperms GAD proteins that bind CaM.

Analysis of the pine genome using the pine GAD cDNA as a probe showed several hybridization bands (Fig. 1c), indicating that GAD could be encoded in pine by several genes, as is the case in angiosperms. Phylogenetic analysis of plant GAD protein sequences situates the pine sequence between two consistent groups (bootstrap values higher than 50%) of angiosperm GADs with putative CaM-binding domains, and separate from a third consistent group that contains two rice and Bryophyta sequences which lack the CaM-binding domain (Fig. 1d).

Production of recombinant GAD and characterization

In order to produce antibodies against pine GAD and to further characterize the GAD protein, the cDNA encoding pine GAD was cloned into the pET30a vector and expressed as a poly-histidine-tagged protein in *Escherichia coli* BL21 cells. The recombinant protein was induced in

bacterial cultures at 37°C in the presence of IPTG. Protein extracts from transformed cells were prepared and fractionated by affinity chromatography using Ni-NTA columns. After washing, bound proteins were removed by increasing the imidazole concentration from 10 to 250 mM. Analysis of the purified preparation by SDS-PAGE followed by Coomassie blue staining revealed the presence of a unique band with an apparent size of 56 kD corresponding to the pine recombinant GAD (Fig. 2a). A preparation of the purified protein was injected into rabbit to raise an antiserum against pine GAD. The specificity of the antiserum was tested with protein extracts of transformed bacteria cells. No polypeptide signals were observed in Western blots in control, non-transformed bacterial cultures, or in transformed-cells incubated without IPTG; in contrast, a reactive

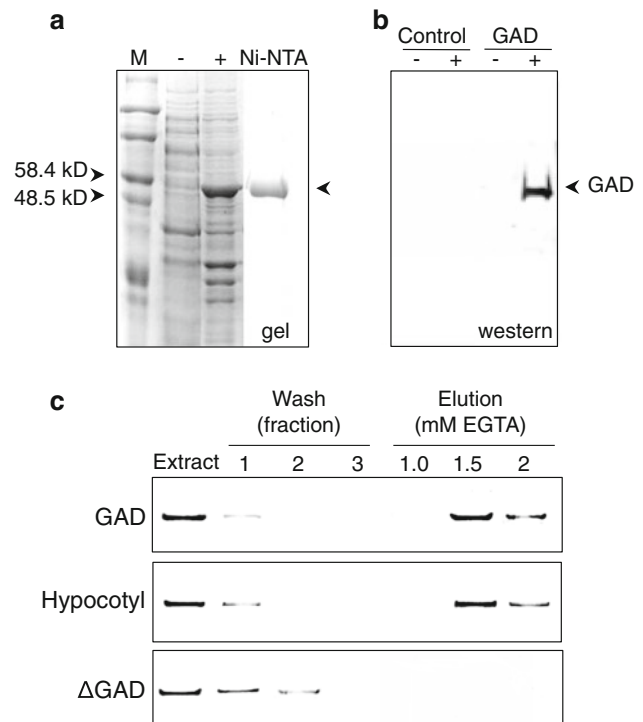


Fig. 2 Expression of recombinant pine GAD in *E. coli* and interaction with bovine CaM. **a** Purification of recombinant pine GAD from *E. coli*. Proteins from non-induced (–) or IPTG-induced (+) cultures were fractionated by SDS-PAGE and subjected to Coomassie blue staining (gel). A unique polypeptide of 56 kD was observed during purification of recombinant GAD through a Ni-NTA column (arrow). **b** Western-blot analysis of protein extracts prepared from control (Control) or transformed (GAD) bacteria cells. The extracts were prepared from non-induced (–) or IPTG-induced (+) cultures. The antiserum was able to recognize the recombinant GAD protein. **c** Bovine CaM-agarose chromatography of protein extracts prepared from bacterial cells producing the recombinant GAD protein (GAD) and from pine hypocotyls (hypocotyl). The same experiment was performed with a truncated form of GAD (Δ GAD) lacking 94 residues at the C-terminal end. After application of the protein extracts and washing, bound proteins were removed with EGTA, separated by SDS-PAGE, and probed in blots with the antiserum against pine GAD

band of 56 kDa was observed in transformed cells when protein expression was induced by IPTG, indicating an adequate specificity of the antiserum (Fig. 2b). According to preliminary experiments, a dilution of 1:20,000 was selected for the detection of GAD protein from bacterial or plant extracts. To determine if the recombinant protein was active, bacterial protein extracts were assayed *in vitro* for GAD activity. No GAD activity was detected in bacterial extracts or purified preparations assayed at pH 7.0 in the absence or presence of Ca²⁺/CaM, suggesting that the poly-histidine tag of the recombinant protein affected GAD activity at pH 7.0. When GAD activity was assayed at pH 5.8 very low levels (2–7 nkat/mg protein) were observed in extracts prepared from non-transformed cell cultures, but a specific activity of 102.1 ± 9.0 nkat/mg protein was observed in the extracts prepared from IPTG-induced transformed cells. The addition of bovine CaM and Ca²⁺ to the reaction mix caused an activation of GAD activity of 32%. These data indicate that bovine CaM and Ca²⁺ partially activate pine GAD at pH 5.8 as it has been observed for recombinant petunia and soybean GADs (Snedden et al. 1995, 1996).

To obtain proof of the interaction between pine GAD and CaM, protein extracts prepared from transformed bacteria cultures expressing the recombinant pine GAD and from pine hypocotyl were fractionated through a bovine CaM-agarose column (Fig. 2c). The recombinant GAD protein was bound to the column and recovered by elution with 1.5 mM EGTA (Fig. 2c, GAD). The elution of GAD from pine hypocotyl extract was identical to the recombinant GAD, and most of the enzyme was eluted from the agarose-CaM column with 1.5 mM EGTA. As a control, a truncated version of the recombinant GAD lacking 93 residues from the C-terminal end was also expressed in bacteria cells, and extracts were fractionated through an agarose-CaM column. As shown in Fig. 2c, the truncated GAD protein, which lacks the putative CaM-binding domain, did not bind to agarose-CaM, and it was directly recovered in the first two fractions during the washing of the column. These results and the prediction of the secondary structure shown in Fig. 1b suggest that the pine GAD can bind CaM *in vivo* as has been suggested for most of the GAD proteins in angiosperms (Bouche and Fromm 2004).

GAD and GABA levels in pine seedling organs

Analysis of GAD expression was carried out in protein extracts from different organs of pine seedlings. GAD activity was quantified at pH 5.8 and 7.0. In all extracts, GAD specific activity was higher in acidic than in neutral pH (Fig. 3a) as has been previously reported for angiosperm GADs (Snedden et al. 1995, 1996). The highest activity was found in the root (1.34 ± 0.06 nkat/mg protein), low

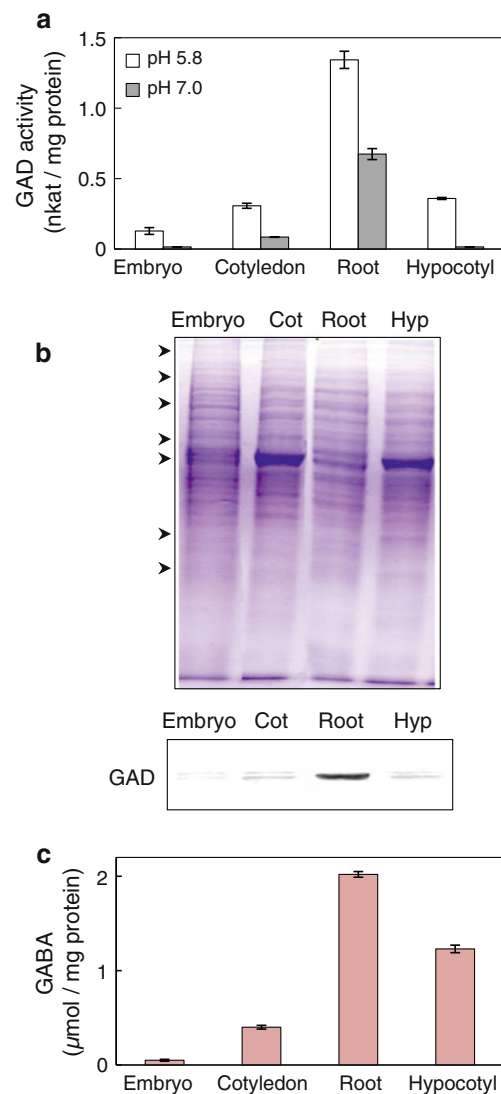


Fig. 3 GAD and GABA levels in pine seedling organs. Protein extracts were prepared from different seedling organs and from the embryo. **a** GAD specific activity was quantified at pH 5.8 and 7.0. **b** Fractionation of proteins by SDS-PAGE (*top*) and Western blot showing GAD polypeptide in pine organs and embryo (*bottom*). Twenty micrograms of protein was subjected to SDS-PAGE, blotted to a nitrocellulose sheet, and probed with antisera raised against recombinant pine GAD. Position in the gel of protein markers corresponding to 180, 116, 85, 58, 48.5, 36.5, and 26.6 kDa are indicated on the left by *arrows*. Apparent molecular mass of GAD polypeptide in Western blots was 56 kDa. **c** GABA levels determined in extracts. Data represent the mean of at least three independent determinations \pm the standard error

levels were detected in germinating embryos (0.13 ± 0.02 nkat/mg protein), and intermediate levels were found in cotyledon and in the hypocotyl (0.31–0.36 nkat/mg protein; Fig. 3a). Analysis of GAD protein was carried out by Western blot using the antisera raised against the recombinant pine GAD. The steady-state levels of GAD polypeptide closely matched the GAD activity values, and the signal was lower in the germinating embryo, cotyledon, and

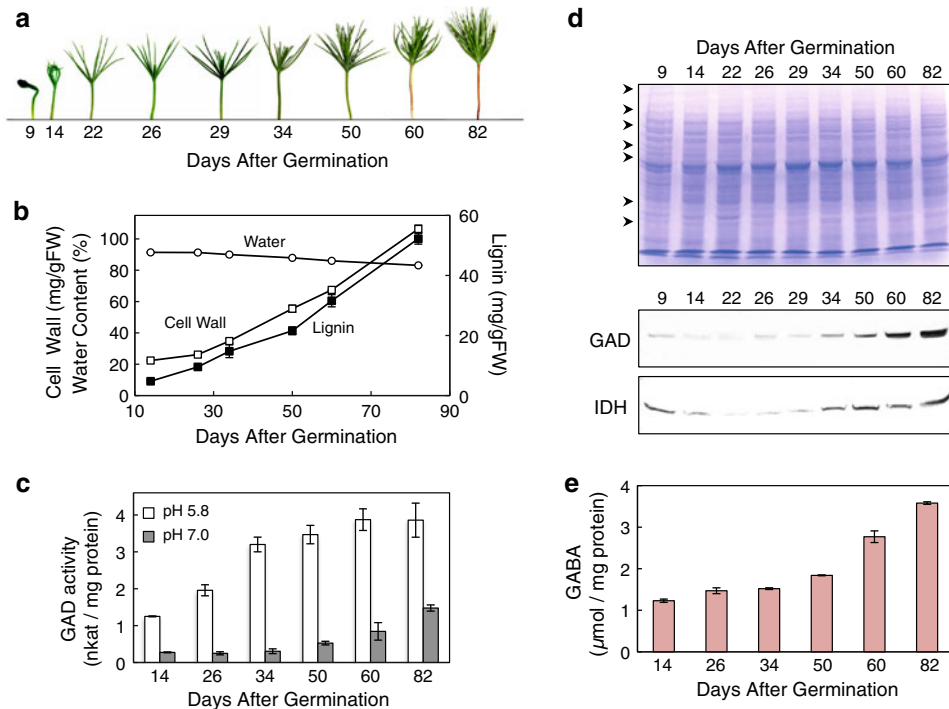


Fig. 4 GAD and GABA levels during hypocotyl development in pine. **a** Representative pictures of the pine seedlings used in this study. **b** Cell wall, water, and lignin content in the hypocotyl of pine seedlings. **c** GAD activity in protein extracts at pH 5.8 and 7.0. **d** Fractionation of proteins by SDS-PAGE (*top*) and GAD and NADP⁺-IDH protein levels as determined by Western-blot analysis using polyclonal antisera raised against the pine proteins (*bottom*).

Position in the gel of protein markers corresponding to 180, 116, 85, 58, 48.5, 36.5, and 26.6 kDa are indicated on the left by arrows. Apparent molecular mass of GAD and NADP⁺-IDH polypeptides in Western blots was 56 and 46 kDa, respectively. **e** GABA levels in the hypocotyl expressed on a protein content basis. Data represent the mean of at least three independent determinations \pm the standard error

hypocotyl than in the root (Fig. 3b). These results are similar to the data available from angiosperms which showed high activity or high protein expression in roots (Chen et al. 1994; Snedden et al. 1995; Turano and Fang 1998). The levels of GABA were also analyzed in pine seedling organs. On a protein content basis, GABA levels correlated to GAD activity and polypeptide signal in Western blot; a low GABA content was detected in the embryo, while the highest levels were observed in seedling roots (Fig. 3c). These data indicate that GABA levels correlate to GAD expression in pine seedling organs.

GAD expression during vascular development in pine

The relative abundance of GAD cDNA clones in EST libraries prepared from the cambial zone of pine and poplar (Cantón et al. 2004; Sterky et al. 2004) could be indicative of a possible role for GAD or GABA in the differentiation of the stem in woody plants. To test this hypothesis, we investigated GAD activity and protein expression as well as the levels of GABA in the hypocotyls of pine seedlings from 9 to 82 days after germination (Fig. 4a). The development of the seedling is intimately associated with the differentiation

of vascular elements. Thus, pine hypocotyl clearly shows secondary vascular development 50 days after germination (data not shown), and its differentiation process is concomitant with a decrease in the water content and the accumulation of extractable cell wall and lignin in the organ (Fig. 4b). The profile of GAD-specific activity was related to the lignification of the hypocotyl, reaching peak values of activity at pH 5.8 and 7.0 at the end of the period studied (Fig. 4c). The amount of GAD polypeptide was also studied by Western-blot analysis. As described previously in the seedling organs, the levels of GAD polypeptide closely matched the GAD activity during the differentiation process (Fig. 4d). A similar pattern of protein expression was also observed for NADP⁺-IDH, one of the main enzymes involved in the synthesis of 2-oxoglutarate, the precursor of glutamate (Fig. 4d). NADP⁺-IDH has been reported to be linked with hypocotyl differentiation in pine (Palomo et al. 1998). Accordingly, these data suggest that GAD, like NADP⁺-IDH, is connected with the differentiation of the hypocotyl in pine. The GABA levels in the organ, based on protein content, also matched GAD expression (Fig. 4e), suggesting that GAD activity is the major regulator of hypocotyl GABA levels during seedling development.

To further characterize GAD in pine, we compared GAD expression with the expression of other genes encoding enzymes associated with seedling differentiation, including cytosolic NADP⁺-IDH (Palomo et al. 1998; Pascual et al. 2008), ascorbate peroxidase (APX), and oxidase (AOX) (Pignocchi and Foyer 2003; de Pinto and De Gara 2004), enzymes linked with lignification and signaling, including laccase (LAC, Gavnholt and Larsen 2002), and 1-aminocyclopropane-1-carboxylic acid synthase (ACS), and oxidase (ACO), enzymes involved in ethylene synthesis that has been described to be connected to lignification of plant stem (Klintborg et al. 2002). Northern blots hybridized with specific probes indicated that these genes were differentially expressed in organs of pine seedlings (Fig. 5a). GAD showed higher expression in root than in hypocotyl, and very low levels in cotyledon (Fig. 5a), which is consistent with previous observations at protein and activity level (Fig. 3a, b). A similar pattern, with very low expression in cotyledons, was detected for the signals corresponding to NADP⁺-IDH, ACS, and ACO expression (Fig. 5a). In contrast, the patterns of expression for APX, AOX, and LAC in seedling organs were different from those of GAD. APX and AOX mRNA signals were clearly detected in cotyledons, whereas LAC expression was high in hypocotyl and low in root and cotyledon (Fig. 5a). These differences in the observed expression profile possibly reflect differences in the differentiation state of the seedling organs and different roles of the genes encoding these proteins in them. When their expression profiles were followed during hypocotyl development, all exhibited a related pattern that correlated to the lignification of the seedling, showing peaks of expression at 60 or 82 days after germination (Fig. 5b). These data are indicative that the expression of these genes is associated with the differentiation of the hypocotyl in pine.

In order to determine if the expression of GAD and other genes are also associated with the differentiation of vascular elements in pine stem, we induced the production of reaction wood by bending 6-month-old pine seedlings for 30 days. Reaction wood is typically produced in trees in response to a mechanical stress to help to position newly formed parts of the plant in an optimal upright position. In conifers, the reaction wood corresponds to a compression wood that is developed below the bend and results from higher lignification and the production of a xylem of compression (Timell 1986). Typically, genes related to xylogenesis and vascular differentiation are expected to show a differential expression during the formation of reactive wood (Plomion et al. 2001 and references therein).

As depicted in Fig. 6a, the mechanical stress of pine seedlings produced an eccentric radial growth of the stem largely due to the development of new xylem vessels in the compression zone. The new structure was not observed in

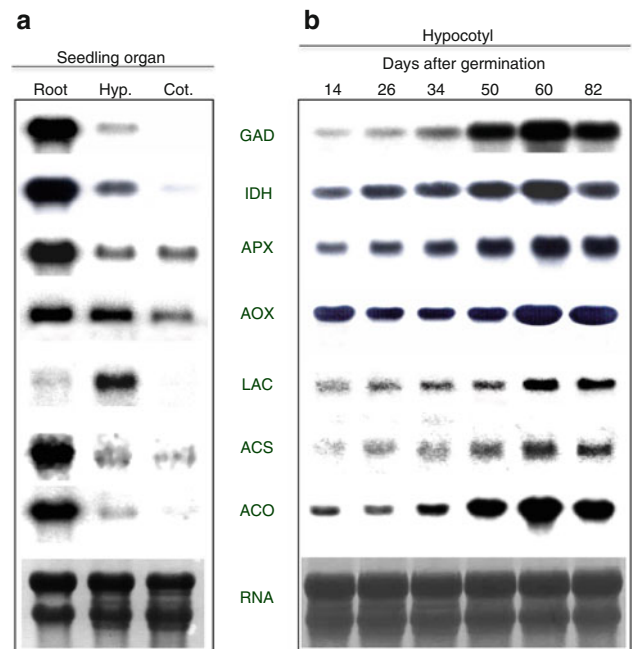


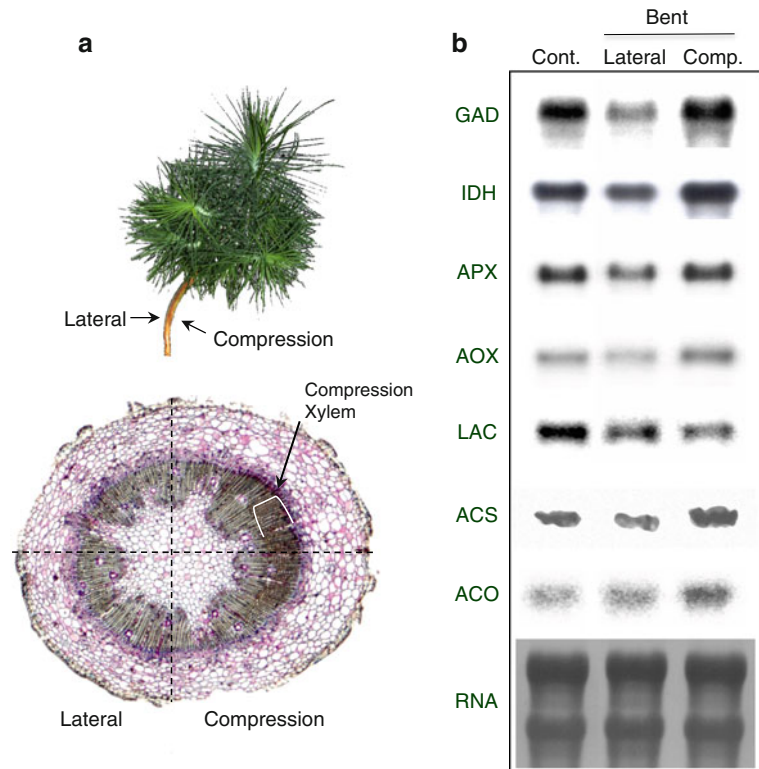
Fig. 5 Gene expression analysis in pine seedling organs (a) and during hypocotyl development (b). Total RNA extracts were prepared, and gene expression was analyzed by Northern-blot analysis using specific probes. Ten micrograms of RNA was loaded per lane. RNA panels correspond to methylene blue staining of the filter

the lateral (opposite) zone above the bent part (Fig. 6a). RNA extracts from the lateral and compression zone were prepared and analyzed by Northern blot with specific probes (Fig. 6b). The mechanical stress affected the expression of all genes considered in this study. GAD and NADP⁺-IDH mRNA levels were higher in the compression zone compared with the lateral zone (Fig. 6b). The expression of these genes was also slightly higher in the compression zone than in control, non-bent plants; in the lateral zone, the signal was lower than in control seedlings, also suggesting a down-regulation of gene expression in the lateral zone. A related pattern was observed for APX, AOX, ACS, and ACO, suggesting that the expression of these genes is also associated with the production of reaction wood. The pattern of LAC expression was different; it was down-regulated in both the compression and lateral zones compared with control non-stressed seedlings (Fig. 6b).

Discussion

The reaction catalyzed by GAD is considered to be the main route for GABA biosynthesis in herbaceous plants (Bouche and Fromm 2004). Although GABA can also be produced from the degradation of polyamines (Fait et al. 2008), the GABA levels in pine embryo and organ seedlings closely matched both GAD activity and protein expression (Figs. 3, 4), suggesting that the GAD reaction

Fig. 6 Gene expression analysis in the hypocotyl of control and bending pine seedlings. Six-month-old plants were bent for 1 month, and total RNA was prepared from the compression and lateral halves. **a** Representative picture of a bent pine seedling, and histological section stained with H-E showing the asymmetrical growth of the hypocotyl (barrel axes). The appearance of the compression xylem is indicated. **b** Northern-blot analysis showing expression of GAD and other genes in control (non-bent) plants and in the compression, and lateral zone of bent plants. Ten micrograms of RNA was loaded per lane



can also be considered the main route for GABA biosynthesis during seedling development in pine. The molecular composition of the plant GAD holoenzyme is not clear. Previous studies in angiosperms have shown that GAD has two distinct domains; a well-conserved domain corresponding to the N-terminal a central portion of the protein that is involved in catalysis and binding of the cofactor pyridoxal-5'-phosphate, and a less-conserved C-terminal region that includes a CaM-binding domain involved in the regulation of enzyme activity (Fig. 1, Suppl. Fig. S1). The typical sequence of a CaM-binding domain has been only reported in the GADs of angiosperms; it is absent in GAD sequences from other kingdoms, in two GAD sequences from *O. sativa* (Akama et al. 2001; Akama and Takaiwa 2007), and in the full-length cDNA sequences from Bryophyta (Fig. 1). The *P. pinaster* sequence reported here (Fig. 1) is the first complete GAD from gymnosperms to be described. Pine GAD appears to be encoded by several genes, suggesting that GABA synthesis may be required for different processes in pine, as is suspected in angiosperms. The primary sequence of the pine GAD exhibits similar characteristics to angiosperms, including a 28-residue region with conserved residues and a predicted secondary structure for a CaM-binding domain (Fig. 1). The possible regulation of pine GAD by CaM is supported by the partial in vitro activation of recombinant pine GAD with Ca^{2+} /bovine CaM and by its binding to an affinity CaM-column (Fig. 2). These criteria have been used to characterize other plant

GADs that bind CaM (Arazi et al. 1995; Snedden et al. 1995, 1996; Yevtushenko et al. 2003) and a yeast GAD with a very low degree of conservation with the typical plant CaM-binding sequence (Coleman et al. 2001). Although the phylogenetic analysis of plant GADs situates pine GAD close to its angiosperm counterparts, bootstrap analysis indicated that the pine sequence cannot be consistently grouped within angiosperms (Fig. 1). This result occurs because of the general low degree of conservation within the C-terminal region of GADs which contains the CaM-binding domain (Fig. 1, Suppl. Fig. S1). The existence of many genes encoding CaM in plant genomes could explain this variable region in GAD proteins; the low degree of conservation could be an adaptive response for maintaining regulation by divergent CaMs during evolution.

The study of GAD expression was carried out during the development of pine seedlings, a model that has proven to be useful in understanding the role of enzymes involved in nitrogen and carbon metabolism enzymes in gymnosperms (Pascual et al. 2008). Low GAD levels were observed in dry (Fig. 3) or germinating embryos (data not shown), differing from the expression pattern of other enzymes that are considered to be key in carbon and nitrogen metabolism, such as glutamine synthetase or cytosolic NADP⁺-IDH, which are typically induced during germination and associated with the input load of amino acids for the production of new plant structures (Palomo et al. 1998;

Pascual et al. 2008). Accordingly, it could be suggested that GAD does not have a primordial role at the early stages of pine seedling development. In contrast, the high GAD and GABA levels found in root (Fig. 3) and hypocotyl during differentiation (Fig. 4) indicate either general or specific roles in these organs that have yet to be described.

A high level of GAD expression in roots has also been observed in angiosperms adapted to different natural habitats (Chen et al. 1994; Snedden et al. 1995; Turano and Fang 1998), and GABA has been reported to activate ionic transport in the root (Kinnersley and Lin 2000) and modulate nitrate uptake (Barbosa et al. 2010), suggesting a role in nutrient and mineral acquisition from soil. Therefore, high GAD expression in roots could be considered to be an ancient feature of vascular plants. In fact, GABA synthesis is induced in response to cytosolic pH reduction as result of the optimal acidic pH of GAD activity (Carroll et al. 1994; Crawford et al. 1994), and it has been thought that acidic pre-soils resulting from a high atmospheric concentration of CO₂ are a probable context for the early diversification and evolution of vascular plants (Willis and McElwain 2002). Accordingly, high GAD levels in roots could have been a relevant feature for adaptation to acidic soil during early land colonization of plants. Conifers typically grow in acidic soils where ammonium is the predominantly available nitrogen (Cánovas et al. 1998). Because the incorporation of ammonium ions usually leads to acidification of the root (Gallardo and Cánovas 2002), high GAD levels could help in both the regulation of pH and nitrogen uptake in pine roots. In addition to the above hypothesis, the high expression of GAD and other marker genes in pine roots can also be the result of the root's advanced developmental state compared with the hypocotyl of the seedlings. Indeed, a vascular cambium in the root is already observed in 22-day-old seedlings (unpublished author's data), hinting at its possible involvement in differentiation and initiation of the secondary growth of the root.

The expression analyses support the idea that GAD is involved in development and differentiation of the stem of pine seedlings. Thus, GAD activity, protein, and mRNA levels during hypocotyl development are similar to the expression levels of cytosolic NADP⁺-IDH, an enzyme associated with hypocotyl development and vascular tissue (Figs. 4, 5; Palomo et al. 1998; Pascual et al. 2008), and these genes also exhibited a similar expression pattern during the induction of reaction wood in pine (Fig. 6). The production of 2-oxoglutarate by cytosolic NADP⁺-IDH has been considered as a limiting factor in the biosynthesis of glutamate in plants (Hodges 2002), and the alteration of the glutamate/GABA ratio has been shown to affect the elongation of stem internodes in tobacco and rice seedlings (Baum et al. 1996; Akama and Takaiwa 2007). Therefore, regulation of the synthesis of glutamate and GABA can play a

role in development and differentiation of the stem in plants.

The accumulation of GAD and NADP⁺-IDH transcripts during hypocotyl differentiation in pine (Figs. 4, 5) is paralleled by other genes related to cellular differentiation, such as APX and AOX (de Pinto and De Gara 2004; Passardi et al. 2004), as well as the genes for ACS and ACO, which are involved in ethylene synthesis. Previous reports have described that GABA can stimulate ethylene production in sunflower (Kathiresan et al. 1997), and both GABA and ethylene interact to promote the differentiation of the stem of *Stellaria* seedlings (Kathiresan et al. 1998). Because hypocotyl and stem development in pine are intimately related with vascular differentiation and xylem lignification, the putative role of genes in the differentiation of the vascular system can be determined by studying their expression during the stimulation and production of reaction wood which helps branches and stems recover an optimal upright position after a mechanical stress. In gymnosperms, the reaction wood produces eccentricity in the stem due to the differentiation of a compression xylem (Timell 1986; Fig. 6). GAD and NADP⁺-IDH expression are enhanced in the compression zone, and down-regulated in the lateral (opposite) zone with respect to non-stressed plants (Fig. 6), a pattern also observed for other genes associated with hypocotyl development, including APX, AOX, ACS, and ACO (Fig. 6). Related results have been described for APX, ACO and ACS in pine (Plomion et al. 2000; Barnes et al. 2008) and for ACS and ACO in poplar, in which ethylene is considered to be an important signal in the control of the production of reaction wood (Love et al. 2009). The specific role of GAD or GABA in this context is not yet clear. According to previous studies, GABA could have a signaling role in stimulating hypocotyl and stem differentiation in the cambial region, as described in other processes in plants (reviewed in Bouche and Fromm 2004), or could simply regulate ethylene production, as indicated in the works of Kathiresan et al. (1997, 1998). In addition, GABA could also be produced in differentiating vascular tissue by short-term regulation of GAD activity in response to the vacuolization of the cytoplasm and an influx of Ca²⁺, factors that are strongly linked to the differentiation of tracheary elements (Groover and Jones 1999). Additional work will be necessary to define a possible role for GABA and the specific GAD genes in the differentiation of the vascular elements, and in the putative crosstalk with ethylene in signaling vascular and stem differentiation in trees.

In conclusion, our results suggest that GAD is the main source of GABA in pine seedlings. GAD protein is expressed in the hypocotyl and stem in seedlings and has similar properties to the angiosperm GADs, including its possible regulation *in vivo* by Ca²⁺/CaM and pH. GAD expression is associated with the development of the

hypocotyl in pine seedlings and exhibited a similar expression pattern to other genes related to vascular tissues and differentiation including NADP⁺-IDH, APX, AOX, ACS, and ACO. Further research is needed to describe the role of GAD and GABA in vascular development and secondary growth, including the possible co-regulation of GABA with 2-oxoglutarate synthesis and crosstalk with ethylene signaling.

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