



Characterization of a tissue-specific and developmentally regulated β -1,3-glucanase gene in pea (*Pisum sativum*)

Peter Buchner¹, Christine Rochat*, Sylvie Wuillème and Jean-Pierre Boutin

Laboratoire de Biologie des Semences, INRA, RD 10, 78026 Versailles cedex, France (*author for correspondence; e-mail rochatc@versailles.inra.fr); ¹current address: IACR Rothamsted, Dept. of Biochemistry & Physiology, Harpenden, Herts. AL5 2JQ, UK

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Abstract

As part of a search for seed coat-specific expressed genes in *Pisum sativum* cv. Finale by PCR-based methods, we identified and isolated a cDNA encoding a β -1,3-glucanase, designated PsGNS2. The deduced peptide sequence of PsGNS2 is similar to a subfamily of β -1,3-glucanases, which is characterized by the presence of a long amino acid extension at the C-terminal end compared to the other β -1,3-glucanases. PsGNS2 is expressed in young flowers and in the seed coat and is weakly expressed in vegetative tissues (roots and stems) during seedling development. It is not inducible by environmental stress or in response to fungal infection. In developing pea flowers the transcript is detectable in all four whorls. In the seed coat the expression is temporally and spatially regulated. High abundance of the transcript became visible in the seed coat when the embryo reached the late heart stage and remained until the mid seed-filling stage. *In situ* hybridization data demonstrated that the expression of PsGNS2 is restricted to a strip of the inner parenchyma tissue of the seed coat, which is involved in temporary starch accumulation and embryo nutrition. This tissue showed also less callose deposits than the other ones. The 5' genomic region of PsGNS2 was isolated and promoter activity studies in transgenic *Medicago truncatula* showed a seed-specific expression. Highest activity of the promoter was found in the seed coat and in the endosperm part of the seed.

Abbreviations: DIG, digoxigenin; GUS, β -glucuronidase; RT-PCR, reverse transcriptase-polymerase chain reaction

Introduction

Plant β -1,3-glucanases represent a highly diverse family of hydrolytic enzymes. This diversity is explained by differences in the regulation of their gene expression, in the chemical and structural feature of their protein and also in their cellular localization. Their exclusive substrates are 1,3-glucans such as callose and laminarin found in the cell walls of plants and fungi. They are generally induced in response to pathogen attack or environmental stress (Kauffman *et al.*, 1987;

Simmons *et al.*, 1992; Cruz-Ortega *et al.*, 1995). A plant defence-related biological function for these hydrolytic enzymes has been suggested based on their co-ordinated induction, together with chitinases, and their inhibitory effect on *in vitro* growth and sporulation of pathogenic fungi (Jach *et al.*, 1995; Mauch *et al.*, 1988a, b; Vogeli *et al.*, 1988). Another proposed defence role of β -1,3-glucanases is the release of elicitors from pathogen cell walls for the induction of the defence response (Keen and Yoshikawa, 1983). Nevertheless, tissue specificity and developmentally regulated non-pathogen-induced expression of β -1,3-glucanase genes have also been reported (Lotan *et al.*, 1989; Memelink *et al.*, 1990; Hird *et al.*, 1993).

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AJ251646 and AJ251199.

β -1,3-glucans are found in several plant structures including the wall of pollen tubes, the sieve plate of phloem elements and endosperm cell walls (Fincher and Stone, 1981). In anthers, β -1,3-glucanase is part of the callase complex and its activity is responsible for the dissolution of the tetrad callose wall and the release of the young microspores into the anther locules (Frankel *et al.*, 1969; Stieglitz, 1977). Recently it was shown that a β -1,3-glucanase protein is involved in somatic embryogenesis process in *Cichorium* (Helleboid *et al.*, 2000). In sieve elements, β -1,3-glucanase activity was correlated with the dissolution of callose plugs (Abeles and Forrence, 1969). The role of this hydrolase in other healthy, unstressed tissues is unclear. In growing plant tissues, the activity of β -1,3-glucanases suggests that they participate in the processes mediating changes in the structure of the cell wall (Kotake *et al.*, 1997). It is also proposed that β -1,3-glucanases are involved in a programmed protection against possible pathogen attack during development.

Regulation of seed development in legumes involves the co-ordinated expression of genes in both the seed coat (maternal origin) and the embryo. Studies of genes specifically expressed in the seed coat during seed development may provide further clues to better understand the role of this organ in embryo development. To investigate this, we have isolated and characterized a cDNA encoding a β -1,3-glucanase from pea (*Pisum sativum*). The data we report on this new pea β -1,3-glucanase isoform shed additional light on the structural differences of this diverse family of hydrolases and their possible evolution. Detailed gene-expression studies by northern analysis and *in situ* hybridization provide new data relevant to the possible functional role of this novel β -1,3-glucanase isoform, especially during seed development. The isolated 5'-upstream regulatory DNA region was used in a transgenic experiment, and first results indicated that it could be an interesting tool for transgenic approaches to manipulate seed development.

Material and methods

Plant material

Plants of the legume *Pisum sativum* L. cv. Finale were grown in growth chambers under a light/temperature regime of 16 h light/20 °C (300 μ mol photon $m^{-2} s^{-1}$) and 8 h dark/15 °C. Plants were watered daily with a complete N-rich nutrient solution

(10.2 mM NO_3^- , 0.9 mM PO_4^{2-} , 1.8 mM NH_4^+ , 5.2 mM K^+ , 0.8 mM Mg^{2+} , 3.1 mM Ca^{2+} with micro elements per litre: boric acid 2 mg, $MnSO_4$ 2 mg, $ZnSO_4$ 0.25 mg, $(NH_4)_2MoO_7$ 50 μ g, FeEDTA 0.9 mg, pH 5.8). Pods at different developmental stages, as well as other parts of the plant, were harvested in the middle of the light phase. Pods were immediately chilled on ice and used for experiments within 2–3 h. Alternatively, the pod wall tissue, the seed coat and the cotyledons were frozen in liquid nitrogen and stored at -70 °C. Developmental stages were defined as days after flowering or as seed weight. Non-seed material was directly frozen in liquid nitrogen after harvesting.

For stress induction assay by wounding, fully developed leaves were punctured about 30 times with a needle and then harvested 24 h and 48 h later. For salicylic acid treatment, 50 ml of 10 mM salicylic acid solution were applied as an aerosol and leaves were harvested 24 h and 48 h later.

For the expression analysis during seedling development, pea seeds were surface-sterilized by sodium hypochlorite treatment. After incubation in sterile water overnight in darkness, the seed were sterile-germinated on towel paper sheets wetted in liquid nutritive solution in the culture chamber under a 16 h/8 h light/dark regime at 20 °C. At 5, 7, 12, 15 and 20 days after watering, seedling stems including primary leaves, roots and also cotyledon of two seedlings were separated and directly frozen in liquid nitrogen and stored at -70 °C until RNA isolation.

For aluminium toxicity assay, 7-day old seedlings were incubated on paper towel sheets wetted with basic nutrient solution containing 200 μ M $AlCl_3$ at pH 4.4–4.8 (HCl). Parallel seedlings were cultivated on basic nutrient solution at pH 4.4 and pH 6.0 (HCl). To guarantee a constant pH it was controlled every 6 h and the nutrient solution was changed as soon as a pH change was detected. Roots of 2 seedlings were harvested after 24 h and 48 h and directly frozen in liquid nitrogen and stored at -70 °C.

For pathogen inoculation, 7-day old seedlings were processed as described by Chang *et al.* (1992). The seedlings were punctured and wrapped in sterile paper towels saturated with sterile distilled water or *Fusarium solani* f.sp. *pisi* macroconidies. The seedlings were incubated at room temperature for 10, 29, 57 and 72 h. Four seedlings were then separated into stems and roots. All tissues were frozen in liquid N_2 and stored at -80 °C.

PCR-mediated cloning of cDNA and sequencing

For the isolation of 3' cDNA fragments by the 3'-RACE technique the following sense primers derived from the 3' end of an internal partial H⁺-ATPase cDNA fragment from pea were used: ATP1SENSE1, 5'-TGCTGGTGCTCCATGGCAGTT-3'; ATP2SENSE2, 5'-GAATACGGAGGGCACTAAAC-3'; ATP1SENSE2, 5'-CTGGTAGGAGACTTGGAATG-3'. First-strand cDNA synthesis using as template 1 µg total RNA from middle-stage seed coat and 5 pmol adapter primer (Gibco-BRL 3'-RACE system) was performed with Superscript reverse transcriptase II (Gibco-BRL) according to the manufacturer's protocol. Subsequent PCR with *Taq* polymerase (Ampli*Taq*, Perkin Elmer) was performed under standard conditions with 1.5 mM MgCl₂, 0.2 µM of the sense primer and the abridged amplification primer of the 3'-RACE system. The temperature regime was 94 °C for 5 min, 40 cycles of 1 min 55 °C, 3 min 72 °C and 1 min 94 °C, and 72 °C for 7 min. A second PCR was performed under the same conditions with 5 µl of the first PCR reaction and a second primer in nested orientation to the primer, which was used in the first PCR reaction. The incomplete 5' end of the glucanase cDNA was extended by the 5'-PCR-RACE technique (Gibco-BRL 5'-RACE system) based on the sequence on the partial 3' cDNA fragment. To avoid sequence errors by the high error rate of the *Taq*-DNA polymerase, the full-length cDNA was also isolated by RT-PCR with the *Pwo*-DNA polymerase with proof-reading function, based on the sequence of the 5'- and 3'-non-coding region derived from the RACE-cDNA fragments. All amplified DNA fragments were subcloned into Stratagene vector pCR-Script SK(+).

Fragments of cDNA longer than 400–500 bp were prepared for sequencing in both directions using the double-stranded Nested Deletion Kit (Pharmacia LKB). Sequences of DNA were determined by the dideoxy method by hand with the AmpiCycle sequencing kit (Perkin Elmer) and α³⁵S]-dATP.

Preparation of plant nucleic acids and hybridization procedures

Genomic plant DNA from frozen tissue was isolated by a modified method according to Dellaporta *et al.* (1983) and John (1992).

Ribonucleic acid from frozen tissue was isolated, separated on agarose/formaldehyde gels and blotted onto positively charged nylon membrane as described

in detail in Heim *et al.* (1993). The northern hybridization was performed according Church and Gilbert (1984) with prehybridization/hybridization at 65 °C. A ³²P-dCTP-labelled (Feinberg and Vogelstein, 1983) partial 1.4 kb PsGNS2 cDNA fragment, including the 3'-non-coding region, was used as a hybridization probe. For induction after pathogen *Fusarium solani* inoculation in young seedlings, a 1470 bp DNA fragment of the pea β-1,3-glucanase published by Chang *et al.* (1992) (accession number S51479/L02212), including 500 bp of the 3'-untranslated region, was used as hybridization probe for a positive control. After hybridization the membranes were stringently washed and exposed to Kodak BioMax MS films. The comparative quantification was performed based on scanning and further measurement of the intensity of the hybridization signal using analytical software packages. For the control of equal RNA loading, the membranes were stripped and hybridized with a ³²P-labelled 28S-rDNA probe from *Vicia faba*. For Southern hybridization genomic DNA were digested with appropriate restriction enzymes, separated by agarose gel electrophoresis and transferred onto positively charged nylon membrane based on the alkaline blotting procedure (Amersham Hybond N⁺ manual). Hybridization of the Southern membrane was performed as for northern hybridization to specify the PsGNS2-labelled probe.

In situ hybridization

Non-radioactive *in situ* hybridization was performed by a method based on a modified protocol of DeBlock and Debrouwer (1996). Briefly, freshly harvested plant tissue was fixed in 4% formaldehyde/PBS (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl pH 7.4) by vacuum infiltration for 10 min once an hour and overnight incubation at 4 °C after fixative exchange. Dehydration and embedding of the tissue in Paraplast followed the protocol of Jackson (1992), with xylene replaced by HistoClear II. Microtome sections of 10 µm were floated on distilled water (40 °C) and placed on polylysine-coated slides. After overnight incubation at 37 °C, the sections were dewaxed in HistoClear and dehydrated in a graded ethanol series. After proteinase K treatment, glycine blocking and acetylation to block charge interactions that could contribute to non-specific hybridization signals, washing and detection were performed as described by DeBlock and Debrouwer (1996). For DIG-RNA labelling the 1.4 kb PsGNS2-cDNA fragment used for north-

ern hybridization was cloned in plasmid vector pAMP (Gibco-BRL) and linearized before the labelling reaction. RNA labelling by *in vitro* transcription with DIG-UTP was performed according the Boehringer application manual (1996) and the probes were prepared for hybridization as described by DeBlock and Debrouwer (1996) except that no tRNA was used in the purification procedure of the RNA probes.

Callose coloration

Callose staining was performed on paraplant sections using decolorized aniline blue (Dubois *et al.*, 1990) and observed with an Olympus BH-12 microscope with excitation filter (334 nm). Phloem was used as control for the fluorescence of callose.

Isolation of 5'-upstream genomic DNA region of the PsGNS2 gene

The 5'-upstream genomic DNA region of the *PsGNS2* gene was isolated by means of a PCR-based DNA walking system (Universal GenomeWalker kit/Advantage Genomic PCR kit, Clontech) and pea genomic DNA. For the first PCR walk two antisense primers, GlucAs1 (5'-CAACGCCTCGATATCACCGTT-3' and GlucAs2 (5'-TGCCGTCACGTGACGGAGAT-3'), nested in the 5' region of the isolated cDNA were used. For a second PCR walk, two antisense primers, GlucAs3 (5'-TACTGTGTTATTTGTCATGGTATCTCGG-3') and GlucAs4 (5'-ATGGTATCTGGGAGTTTTAGTTGA-3'), based on the sequence of the first isolated genomic 5'-upstream region were used. A 1.5 kb 5'-upstream fragment to be used in transformation experiments were finally isolated by PCR with a sequence-specific sense and antisense oligonucleotide primer and the Advantage polymerase system (Clontech) based on the derived sequence information of the PCR walks. All derived PCR fragments were subcloned into pCR-Script and sequenced in both orientations.

*Chimeric promoter GUS construction and production of transgenic *Medicago truncatula* plants*

A PsGNS2 promoter chimeric GUS construct was constructed by ligation of the 1.5 kb promoter DNA fragment into pBI101 (Clontech) in the sense orientation to the β -glucuronidase gene. The resulting plasmid, the pBI101PsGNS2 promoter, was directly transformed into *Agrobacterium tumefaciens*, strain EHA105, by a modified (CPY instead of YEB

medium) freeze/thaw method based on Höfgen and Willmitzer (1988). Transformation and regeneration of *Medicago truncatula* from leaves were carried out according to Trinh *et al.* (1998), using ecotype R108-1 (Hoffman *et al.*, 1997; kind gift of seeds from P. Ratet and T.H. Trinh). Briefly, leaf explants were vacuum-infiltrated and further co-incubated with *A. tumefaciens* in liquid Schenk and Hildebrandt medium supplemented with acetosyringone (SHMab, Trinh *et al.*, 1998). The explants were then cultivated in the dark for 5 weeks on solid Schenk and Hildebrandt-based medium with 400 mg/l Augmentin (Beecham) to suppress bacterial growth and 40 mg/l kanamycin monosulfate (Sigma) for selection. Transformed proembryogenic calluses further developed in the light. Kanamycin-selected rooted plantlets were further cultivated in soil, and seeds of the first generation were planted for GUS assay studies. All transgenic plants used for GUS assay were analysed by PCR with sequence-specific oligonucleotides corresponding to the PsGNS2 promoter fragment.

Assays for GUS activity in transgenic plants

The fluorometric assay was performed as described by Jefferson *et al.* (1987) on extracts made from different parts of transgenic and control plants. The reaction product of the assay (4-methylumbelliferone) was measured kinetically, in duplicate, using a fluorometer (Labsystems, Fluoroskan II, Helsinki, Finland). Protein concentrations of extracts were determined by the method of Bradford (1976). To allow comparison for all transgenic plants, absolute activities per protein unit were converted in relative units: for each individual plant, activity measured for entire young seed was used as 100% level and activities measured in other organs were converted on this basis. This allows us to compare relative expression levels of the reporter gene.

Histochemical localization of GUS activity was performed with 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-Gluc) as a chromogenic substrate (Jefferson *et al.*, 1987). Different tissues from control and transgenic plants were stained at 37 °C in 2 mM X-Gluc in 0.2 M phosphate buffer. For studies on seeds, 20% methanol was added to the reaction medium to suppress endogenous GUS-like activities as suggested in Kosugi *et al.* (1990). Sections or organs were then cleared by washes in 70% methanol. Sections of the GUS-stained plant tissues were analysed microscopically.

Results

The cDNA derived sequence structure of a newly isolated β -1,3-glucanase

The 3'-cDNA fragment isolation of a H^+ -ATPase by the RACE techniques, using degenerated primers, applied to a preparation of RNA from pea seed coats led also to the amplification and identification of other, non-ATPase, cDNA fragments. The expression of the corresponding genes of these fragments was analysed by northern hybridization, and one fragment exhibited high expression in the seed coat. The sequence analysis of this partial 1486 bp cDNA fragment, by screening the EMBL databases, revealed similarities to plant β -1,3-glucanases and β -1,3-1,4-glucanases. A full-length cDNA clone was isolated by 5'-RACE technique and RT-PCR using a *Pwo*-DNA polymerase with proofreading function. The complete 1567 nucleotide sequence was found to contain a 1362 bp open reading frame encoding a polypeptide of 452 amino acids with a calculated molecular mass of 49 kDa and an isoelectric point of 8.78 (pI/MW software program, ExPASy Server; Wilkins *et al.*, 1998). There was a putative N-terminal signal peptide of 26 amino acids, enriched in neutral and hydrophobic amino acids typical of eukaryotic signal sequences as suggested by von Heijne (1983). The location of the cleavage site was estimated between position 26 and 27 (sequence VTS-IG) by using the SignalP Version 1.1 software (Nielsen *et al.*, 1997). In the 3'-untranslated 193 bp region, no AATAAA poly(A) signal site was detectable.

Comparison of the DNA and deduced amino acid sequences of PsGNS2 cDNA with those in DNA and protein data banks confirmed that it coded for a β -1,3-glucanase. This gene was named PsGNS2 as suggested by Simmons (1994). Highest amino acid identity (51%) to previously characterized glucanases was found to a wheat β -1,3-glucanase (Cruz-Ortega *et al.*, 1995). The identities to other plant β -1,3-glucanases and also β -1,3-1,4-glucanases vary from 36% to 41%. Further phylogenetic analysis (bootstrapped tree of the multiple amino acid sequence comparison based on CLUSTAL W program (Thompson *et al.*, 1994) version 1.7 and Treeview32 program (Page, 1996)), shown on Figure 1A, significantly clustered PsGNS2 into a subfamily together with β -1,3-glucanases from different plant species including wheat, *Arabidopsis*, *Brassica napus*, *Salix gilgiana*, *Olea europea* and tobacco. The members of this subfamily are characterized by the presence of a long amino acid extension

of 112–114 amino acids at the C-terminal end that is absent from other glucanase sequences (Figure 1B).

Tissue specificity and developmental regulation of the expression of PsGNS2

The seed coat origin of the RNA used in the 3'-RACE suggested that the expression of *PsGNS2* may be tissue-specific and/or developmentally regulated. We first characterized the expression of *PsGNS2* in different organs of flowering and seed-developing pea plants. The Southern analysis indicated that the labelled 1.4 kb *PsGNS2* cDNA probe used hybridized only to one gene in the pea genome with no cross-hybridization to other genes under the conditions used for the northern hybridization (data not shown). Northern blot hybridization was performed with total RNA from roots, stem, source and sink leaves, young flower buds before fertilization and also from pod walls, seed coat and cotyledons of developing pea fruits. After hybridization with the partial *PsGNS2* cDNA fragment, a strong hybridization signal was seen in the seed coat and a weak signal in young flower buds (Figure 2A). Further detailed analysis of the expression in young flower buds showed that *PsGNS2* transcript was observed in all four flower whorls, sepals, petals, stamens and carpel, including the ovules, sampled prior to pollination. The signal in petals is very weak whilst in stamens and carpel/ovules a strong signal occurred (Figure 2B). The signal in sepals is only visible after long exposure (not shown). Detailed temporal expression was followed during pea seed coat development (Figure 2C). An increase of the amount of *PsGNS2* transcript was observed during early embryogenesis in seeds whose fresh weight increased from 7 to 30 mg. This maximum level of *PsGNS2* RNA diminished slowly until the seeds developed to the weight of 220 mg (close to the middle of seed filling stage); then the transcript amount dropped drastically and disappeared during further development.

The expression of *PsGNS2* was also checked in pea seedlings. During the first 20 days after watering *PsGNS2* transcript was detectable in the cotyledons, the roots and also in the developing stem including the primary leaves (Figure 2D). In cotyledons only a very weak transcript signal was visible. The stronger signal found in root decreased continuously until day 20. The hybridization signal in the stem did not to change during this period of seedling development. The quantitative measurement of the hybridization signals in the cotyledon, root and stem of the developing seedlings

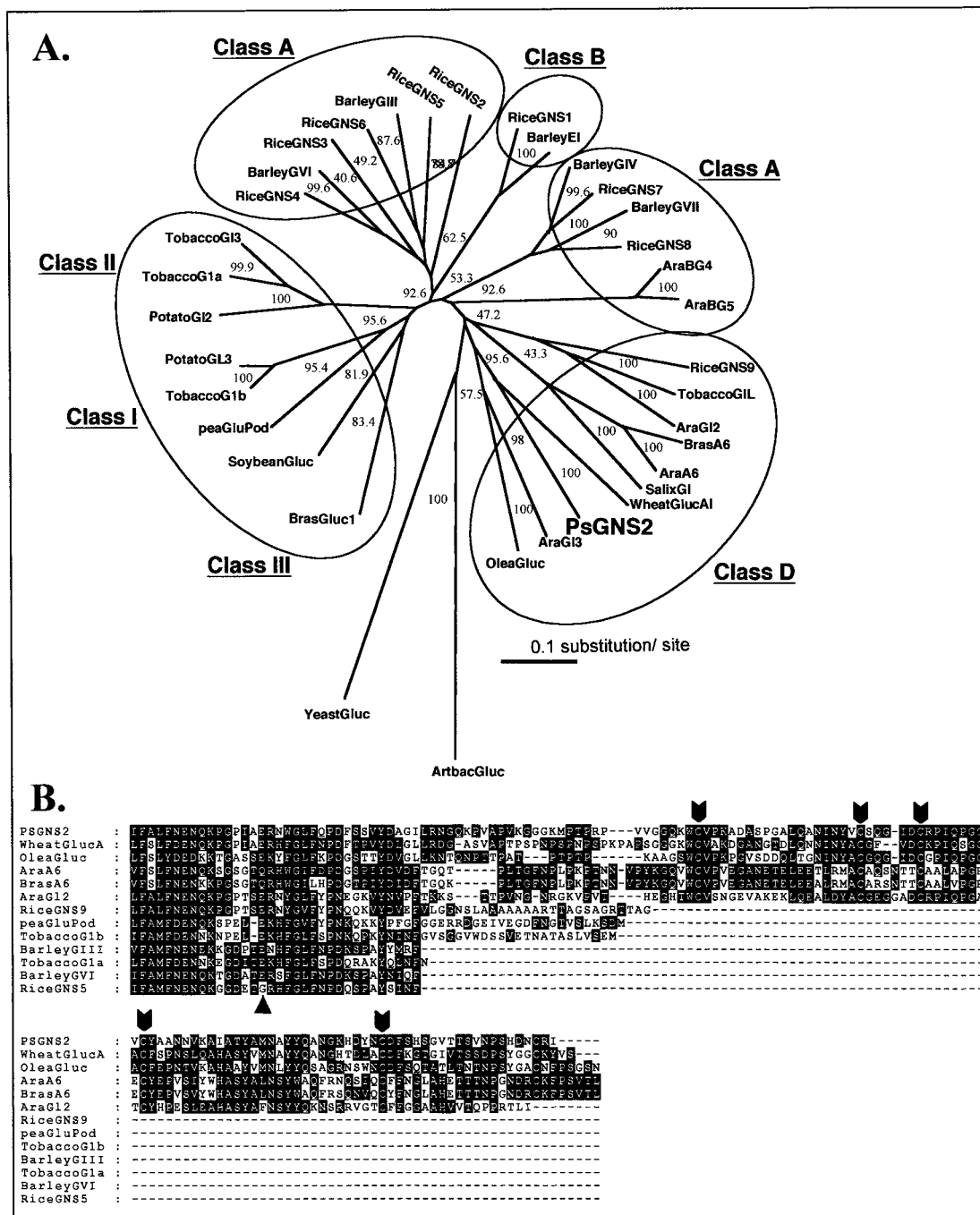


Figure 1. Comparison of plant β -1,3-glucanases and β -1,3,4-glucanases. **A.** Unrooted bootstrapped dendrogram of the alignment of selected β -1,3-glucanases and β -1,3,4-glucanases corresponding to all known classes and subfamilies (Simmons, 1994; Romero *et al.*, 1998) including a yeast and a bacterial β -1,3-glucanase. The putative amino acid sequence of PsGNS2 was aligned with corresponding β -1,3-glucanases and β -1,3,4-glucanases from the monocotyledons: barley (Xu *et al.*, 1992), rice (Romero *et al.*, 1998), wheat (Z22874; Cruz-Ortega *et al.*, 1995). β -1,3-glucanases were from the dicotyledon species tobacco (Simmons, 1994; AB050628.1), *Arabidopsis* (Hird *et al.*, 1993; Delp and Palva, 1999; CAB79538, AAD22313), oilseed rape (Hird *et al.*, 1993; X77990), potato (Beerhues and Kombrink, 1994; AJ009932), soybean (U41323), pea (Chang *et al.*, 1992), olive (Huecas *et al.*, 2001), *Salix gilgiana* (Futamura *et al.*, 2000) and also from yeast (Klebl and Tanner, 1989) and Antherobacteria (D23668). Alignments were performed with the CLUSTAL W program (Thompson *et al.*, 1994) version 1.7 and the tree was drawn with the Treeview32 program (Page, 1996). The % values of bootstraps supporting this topology are shown. **B.** Amino acid sequence alignments of the C-terminal region of selected β -1,3-glucanases. Shaded residues indicate positions where amino acids are highly conserved. The arrows indicate the catalytic acid residues (Glu-288 in isoenzyme BarleyGIIb) based on Høj and Fincher, 1995 and the conserved Cys residues in the C-terminal extension domain.

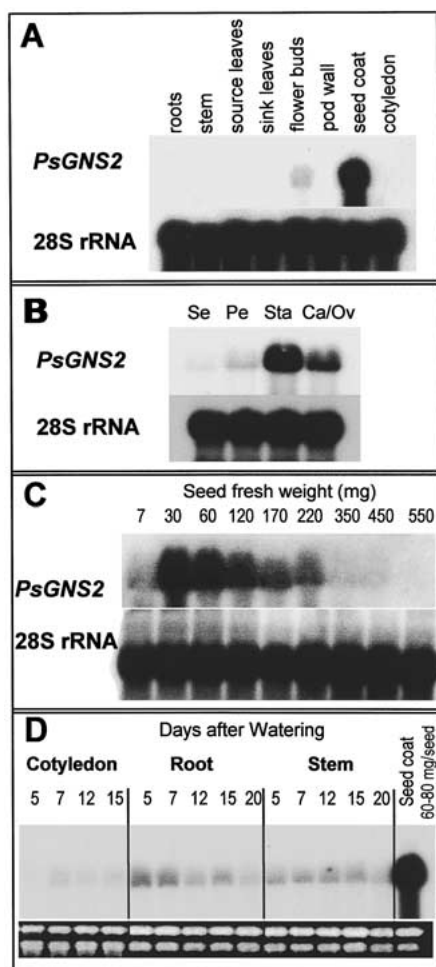


Figure 2. Expression of *PsGNS2* transcript in pea plant. Steady-state levels of *PsGNS2* mRNA in different tissues of pea (A), in the four separated whorls of young flower buds (B), in the seed coat during seed development (C) and in the pea seedlings during watering (cotyledon, stems including primary leaves and roots) (D). Total RNA (10 μ g/lane) was separated in a formaldehyde/agarose gel, transferred onto positively charged nylon membranes and hybridized to a [^{32} P]-labelled *PsGNS2* cDNA fragment. Except for D the same membranes were re-hybridized with a partial [^{32} P]-labelled 28S-rDNA fragment after stripping of the *PsGNS2* probe. In D control of equal loading is shown by ethidium bromide staining of the ribosomal RNA on the membrane after transfer. Se, sepals; Pe, petals; Sta, stamen; Ca/Ov, carpel including ovaries.

compared to the maximal signal found in the seed coat indicated a 57-fold stronger expression of *PsGNS2* in the seed coat compared to that in the young seedling.

PsGNS2 in situ hybridization in the seed coat

The strong temporally regulated expression of *PsGNS2* in the seed coat during seed development was analysed further to determine the spatial expression

pattern in the seed coat by using *in situ* hybridization. The pea seed coat consists of different tissue layers: an outer layer composed of an epidermis and a hypodermis, a parenchymatous tissue which is composed of concentric layers of chlorenchyma, ground parenchyma in which the vascular system is embedded and an inner thin-walled parenchyma or also called transfer tissue, which forms the innermost cell layers of the seed coat (Tegeder *et al.*, 1999). The cellular localization of *PsGNS2* was examined by exposing tissue sections to *in vitro* DIG-labelled antisense and sense RNA probes prepared from the cDNA fragment used for northern analysis. The maximum expression window for *PsGNS2* was chosen (seeds of 40–60 mg fresh weight). A signal could be detected only with the antisense probe (Figure 3A, B) and the strong signal was confined to the inner thin-walled parenchyma, which also surrounds the radicle sheet in which the embryonic radicle is growing during seed development (Figure 3D, E). No hybridization of *PsGNS2* to other tissue cell layers including the vascular bundle was detectable (Figure 3C). Comparison of the hybridization signal to starch granule content (Figure 3F) leads to the conclusion that *PsGNS2* was specifically expressed in the inner thin-walled parenchyma tissue layer where temporary starch accumulation and nutrient transfer to the embryo during seed development occurs (Rochat and Boutin, 1992). We also checked the presence of callose in the pea seed coat. Light micrographs of seed coats from 40–60 mg seed fresh weight show the presence of callose throughout the seed coat (Figure 3G). Callose deposits were present to a great extent especially in the wall of the ground parenchyma cells which showed numerous pit-fields of plasmodesmata (Figure 3H, I). A thin callose deposit was also present in the zone that appears situated at the border of crushed thin-walled parenchyma transfer cells and the endosperm (Figure 3J).

Stress-induced expression analysis of PsGNS2

β -1,3-glucanases, as well as other pathogenesis-related proteins, can frequently be induced either by salicylic acid or wounding (Simmons *et al.*, 1992; Hennig *et al.*, 1993). However, neither treatment of pea leaves with salicylic acid nor wounding induced the expression of *PsGNS2* in pea leaves (data not shown). Expression of the wheat β -1,3-glucanase, closely related to *PsGNS2*, was associated with aluminium toxicity in wheat seedling roots (Cruz-Ortega *et al.*, 1995). To analyse whether *PsGNS2* expression

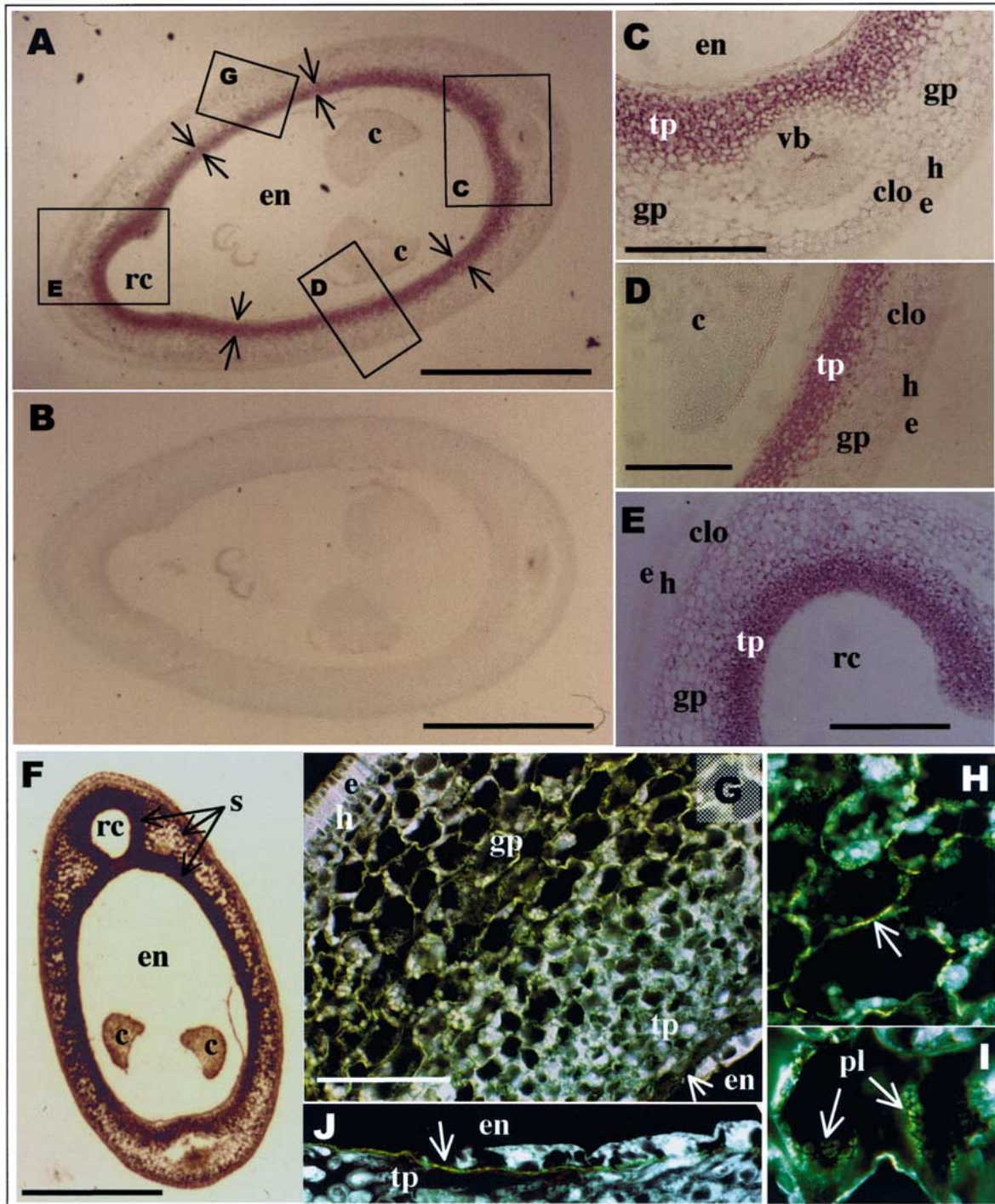


Figure 3. Localization of *PsGNS2* mRNA by *in situ* hybridization, starch grains and callose deposits in cross sections through a pea seed 40–60 mg fresh weight. **A.** Bright-field microscopy after *in situ* hybridization with *PsGNS2* DIG-labelled antisense RNA and detection using alkaline phosphatase-conjugated anti-DIG-Fab fragment and NBT/BCIP as substrate. **B.** After hybridization with *PsGNS2* DIG-labelled sense RNA and detection. **C–E.** Enlarged sections of **A**. **F.** Starch staining with KI on a contiguous cross-section of **A**. **G.** Callose coloration on a contiguous cross-section of **A**. Callose deposits appear as yellow fluorescent patterns (arrows). **H, I.** Enlarged views of **G** showing callose deposits in the wall cell and pit-fields of plasmodesmata in ground parenchyma. **J.** Enlarged view of **G** showing the fine callose deposit (arrow) existing at the border between the thin-walled parenchyma and the endosperm. c, cotyledon section of an embryo at early cotyledon stage; clo, chlorenchyma; e, epidermis; en, endosperm; gp, ground parenchyma; h, hypodermis; pl, plasmodesmata; tp, thin-walled parenchyma = transfer tissue; rc, radicle cave; s, starch deposits; vb, vascular bundle. The bars represent 1 mm in **A**, **B** and **F**, 0.3 mm in **C**, **D** and **E**, and 0.1 mm in **G**.

was also induced under the influence of aluminium, pea seedlings were exposed to levels of aluminium more than two-fold greater than that described for the wheat seedlings treatment (Cruz-Ortega and Ownby, 1993). The hybridization of total RNA from seedlings exposed to aluminium- or non-aluminium-containing medium for various times were compared by northern analysis (Figure 4A). Before aluminium treatment started, a weak transcript signal was visible in seedling roots 7 days after watering. After 24 h or 48 h of aluminium treatment no significant change in *PsGNS2* transcript level compared to the control treatment was visible. On the other hand, the acidic pH (4.6), which ensures the solubility of AlCl_3 , had only a small effect on the expression pattern. For further stress-induced expression analysis pea seedlings were inoculated with *Fusarium solani* f. sp. *pisi* spores as described by Chang *et al.* (1992). The expression of *PsGNS2* was analysed in roots and stems in a 72 h time course inoculation in comparison to the inducible pea β -1,3-glucanase gene (accession number L02212, Chang *et al.*, 1993). As described by Chang *et al.* (1992), we observed a slight transcript increase after 29 h of water treatment in stems and roots of the young seedlings for the chitosan/*Fusarium*-inducible β -1,3-glucanase (Figure 4B). It subsequently disappeared during further water treatment in stem, but not in roots. *Fusarium solani* spore inoculation led to a slight increase 10 h after inoculation in stems and a far higher transcript level in roots compared to initial level. Transcript level remained steady in stems and roots during the whole time course. As already shown in Figure 2D, *PsGNS2* is expressed in roots and stems of the young seedlings but neither H_2O nor *Fusarium solani* infection changed the transcript level significantly during the whole time period of the treatments.

PsGNS2-promoter-mediated *GUS* expression in transgenic *Medicago truncatula* plants

The PCR-based 5' genomic walk with *PsGNS2* sequence-specific oligonucleotide primers resulted in the isolation of 2071 bp of the *PsGNS2* 5'-upstream genomic region. Results of the computer-based analysis of potential transcription factor binding sites (Heinemeyer *et al.*, 1998) is shown in Figure 5. We identified several potential binding sites for the SBF1 factor, which binds to the silencer region of the chalcone synthase promoter (Lawton *et al.*, 1991) and the AthB-1 factor, the homeobox protein 1 from *Arabidopsis thaliana* (Sessa *et al.*, 1993). Further on,

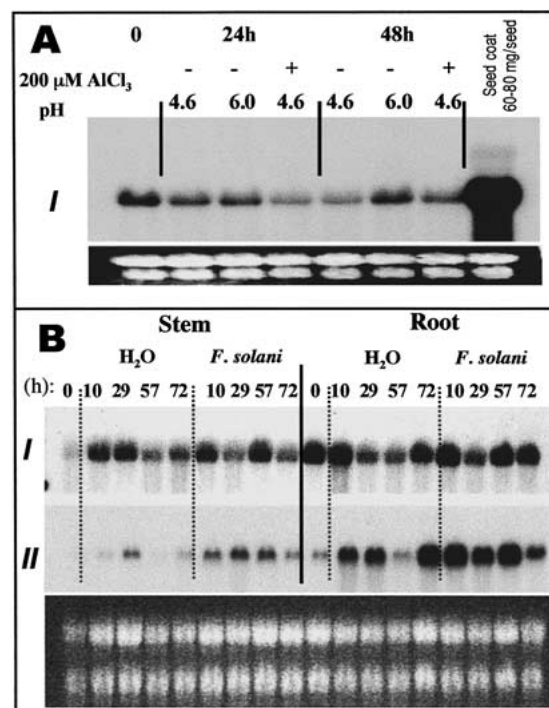


Figure 4. Influence of aluminium and *Fusarium solani* f. sp. *pisi* inoculation on *PsGNS2* transcripts in pea seedlings. A. Expression of *PsGNS2* (I) in roots of seedlings grown in conditions of aluminium toxicity. B. Expression of *PsGNS2* (I) and an inducible pea β -1,3-glucanase (accession number S51479/L02212; II) in tissues of seedlings submitted to *Fusarium solani* infection. The northern hybridization procedure was performed as described in the legend to Figure 2. Equal loading is shown by ethidium bromide staining of the ribosomal RNA on the membrane after transfer.

a potential binding site for G-box binding proteins and for MYB.PH3, a petal-specific transcription factor from *Petunia hybrida* (Solano *et al.*, 1995) was found in the 5'-upstream region of *PsGNS2* (Figure 5).

To assess the capacity of the promoter to specifically direct expression in the seed coat of a legume species, we made use of the model plant *Medicago truncatula*. This legume is phylogenetically close to pea (Doyle, 1999) and can be transformed (Trinh *et al.*, 1998). For the construction of a chimeric *PsGNS2*-promoter *GUS* fusion in pBI101, a 1.5 kb fragment DNA promoter containing 42 bases of the 5'-non-coding region of the *PsGNS2* mRNA was used (Figure 5). *Agrobacterium tumefaciens*-mediated transformation of leaf explant from *M. truncatula* (line R108-1, Hoffmann *et al.*, 1997) was carried out (Trinh *et al.*, 1998). Four independent transgenic lines of *M. truncatula* were selected and further cultivated for T₂ generation. Fluorometric measurements of *GUS* activity on different plant extracts in trans-

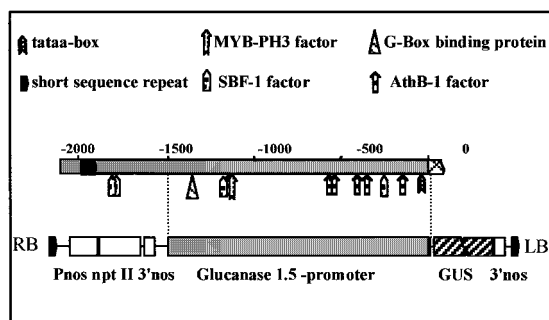


Figure 5. Structure of the *PsGNS2* glucanase promoter. Graphical presentation of the location of potential transcription factor binding sites including a short sequence repeat and the TATAA box in the 5' DNA region of the *PsGNS2* gene, and presentation of the promoter-GUS fusion construct in pBIN used for transformation experiments. Athb1, *Arabidopsis thaliana* homeobox protein (Sessa *et al.*, 1993); MYB-PH3, petal epidermis-specific MYB transcription factor (Solano *et al.*, 1995); SBF1 (Lawton *et al.*, 1991); Pnos, nopaline synthase promoter; nptII, neomycin phospho-transferase gene; 3'-nos, nopaline synthase gene terminator; GUS, β -glucuronidase gene; LB and RB, left and right border regions of the binary vector pBIN.

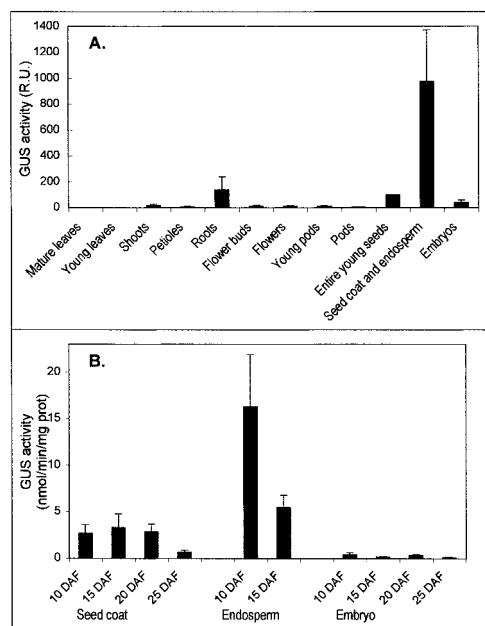


Figure 6. GUS activity in different tissues of transgenic *PsGNS2*-promoter GUS *Medicago truncatula* plants. A. GUS activity in different organs of the transgenic plants. Data represent the relative GUS activity in the plant, and were obtained for 6 individual replicates on individual plants. For each individual transgenic plant, activity measured for entire young seed was used as 100% level and activities of other organs of the plant were converted on this base. B. GUS activity in *Medicago* seed compartments at different developmental stages. Data are the mean of 5 individual measurements on 2 independent lines.

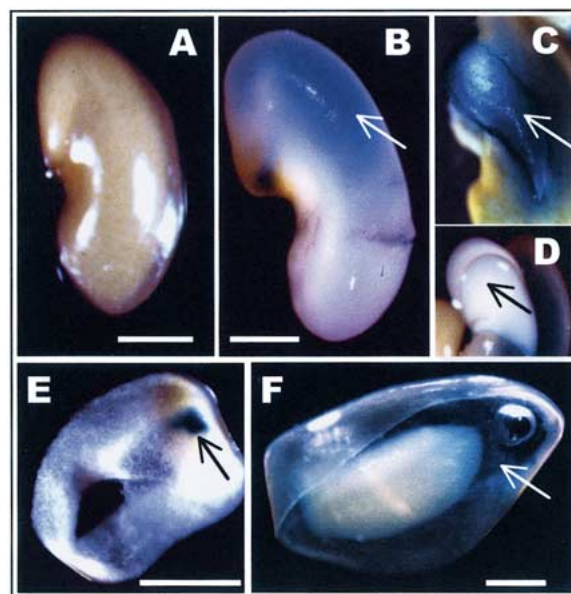


Figure 7. GUS-activity staining in transgenic *Medicago* seeds. A. Wild-type seed. B. Transgenic entire seed. C. Detail showing the strong coloration of the endosperm part of the seed. D. Detail showing the absence of GUS-staining in the embryo. E. Vibratome cutting showing the strong but limited coloration in the empty seed coat. F. Vibratome cutting showing the blue coloration of the gelatinous endosperm in an older seed. The bars represent 1 mm in A, B, 0.5 mm in E, F.

genic *PsGNS2*-promoter-GUS *Medicago truncatula* plants (Figure 6A) showed that a strong relative expression occurred in seed coats and endosperm. No detectable GUS activity was found in either leaf petioles or pod walls and a low background expression occurred in shoots and flower buds. A weak expression was found in very young seeds, embryos and roots. The strong expression in seed organs was confirmed by more detailed measurements of GUS activity during *M. truncatula* seed development (Figure 6B). We were able to separate seed coat, endosperm and embryo by dissection of the seed at given developmental stages and GUS activity was measured for each compartment. GUS activity was principally located in the endosperm compartment of *Medicago* seed; nevertheless, significant expression occurred also in the seed coat. Almost no GUS activity was measured in the embryos. The greatest GUS activities were found in young tissues. As a low expression of *PsGNS2* was detected in pea seedlings (Figure 2D), a study of GUS gene expression was also performed in seedlings of the four transgenic lines. Fluorometric measurements showed that GUS coloration generally did not exceed the background activity existing in the wild type in all

seedling organs (data not shown). The analysis of GUS activity in transgenic *PsGNS2*-promoter-GUS *Medicago truncatula* plants, by *in vitro* GUS staining of different tissues on entire seeds and cuttings, showed strong blue staining in the seed during development (Figure 7A–F). GUS staining was visible near the radicle zone (Figure 7B and E); this observation confirmed the expression in the seed coat. Nevertheless, most of GUS staining was localized to the endosperm (Figure 7B, C, F). The embryo completely lacked coloration (Figure 7D). It was also checked that puncturing of leaves does not induce GUS staining.

Discussion

We have identified a new β -1,3-glucanase gene in pea. This study adds further data regarding the structure of the gene family. Moreover, this gene has a very specific and noteworthy pattern of expression. *PsGNS* is not a defence-related gene and its expression is mainly related with seed development. This is the first report on the expression of a β -1,3-glucanase in the seed coat of a leguminous plant. This work raises new questions about the specific role of a β -1,3-glucanase gene in this seed compartment from a functional point of view. We are faced with a great ignorance of the cell wall composition in the seed coat and this study provides new data about the presence of callose in this organ.

Transgenic assessment of the corresponding promoter of the β -1,3-glucanase in *Medicago truncatula* indicates that the promoter can be used as a tool to express genes in the seed coat/endosperm of leguminous seed, to increase knowledge of their function during seed development in terms of nutrition and regulation of development.

PSGNS2 belongs to a subfamily of β -1,3-glucanases characterized by a long C-terminal elongation

A great deal of information on β -1,3-glucanases from different plant species is now available, and with every newly isolated and characterized gene, the high diversity of this family of glycosyl hydrolases seems to increase. We have isolated and characterized a new β -1,3-glucanase from pea, using seed coats as a starting material. The cDNA isolated encoded a β -1,3-glucanase, *PsGNS2*, a polypeptide of 49 kDa with a calculated isoelectric point of 8.78 and a putative N-terminal signal peptide of 26 amino acids. Phylogenetic analysis closely relates *PsGNS2* to a

subfamily, characterized by a long C-terminal amino acid extension that is absent in other known plant β -1,3-glucanases. The similarity between β -1,3-1,4-glucanases and β -1,3-glucanases is very high. Whilst β -1,3-glucanases have been found throughout the plant kingdom, β -1,3-1,4-glucanases have been found only in monocotyledons. Moreover, the presence of the two amino acids Glu-235 and Glu-297 in *PsGNS2* implicated in the identified catalytic amino acids from barley β -1,3-glucanases, and the absence of the third Asp residue, which seems to be involved only in catalysis by barley β -1,3-1,4-glucanases (Barley EII Asp-284; Chen *et al.*, 1993), provide circumstantial evidence that *PsGNS2* encodes a β -1,3-glucanase.

Former classification separated the β -1,3-glucanases into dicotyledon and monocotyledon classes which also included the β -1,3-1,4-glucanases. The dicotyledon class was further separated into three classes: the basic pI vacuolar class I, the acidic pI secreted class II and the class III β -1,3-glucanases. The monocotyledon class was further separated into four classes (A, B, C, D) according to sequence similarity studies, the third (C) comprising the β -1,3-1,4-glucanases (Simmons, 1994; Romero *et al.*, 1998). The structural comparison of *PsGNS2* and β -1,3-glucanases from *Arabidopsis*, *Brassica napus*, *Salix gilgiana*, *Olea europea* and tobacco to the different groups of the described dicotyledon class, suggested that these dicotyledon genes define a modified class of β -1,3-glucanases together with the monocotyledon class D gene classified by Romero *et al.* (1998) (Figure 1A). Similarly, the *Arabidopsis* BG4 and BG5 genes are also closely related to the monocotyledon class C β -1,3-glucanases (Figure 1A). The dendrogram analysis resulted in a modified subfamily classification of plant β -1,3-glucanases and β -1,3-1,4-glucanases in which dicotyledon β -1,3-glucanases are clustered in the former pure monocotyledon classes C and D.

In dicotyledons some basic β -1,3-glucanases are characterized by short C-terminal extensions. The basic β -1,3-glucanases are known to accumulate in the vacuole and the short C-terminal extension is removed by processing (Sinshi *et al.*, 1988; Van den Bulcke *et al.*, 1989). Glycosylation during processing is thought to facilitate targeting them to the vacuole (Shinshi *et al.*, 1988). The *PsGNS2* C-terminal extension contains only one glycosylation consensus sequence (Asn-xxx-Ser/Thr) compared to 3 and 8 glycosylation sites found in the wheat GlucA1 and the oil seed rape/*Arabidopsis* A6 extension respectively (Hird *et al.*, 1993; Cruz-Ortega *et al.*, 1995). A hint that the

long C-terminal extension of β -1,3-glucanase class D might not be processed was found by the immunochemical analysis of the oilseed rape and *Arabidopsis* A6 proteins by Hird *et al.* (1993). In the *Arabidopsis* genome at least 15 putative β -1,3 glucanases containing the long C-terminal extension could be identified (data not shown). Interestingly, the rice GNS9, also clustered in class D by Romero *et al.* (1998), is structurally distinguished by a shorter C-terminal extension without any sequence identity to that of the other isoenzymes of class D (Figure 1B). The sequence comparison of all known β -1,3-glucanases containing the long C-terminal extension (see a selected collection in Figure 1B) identified in this C-terminus domain 5 conserved cysteine residues which are found in all sequences. This may suggest the presence of at least 2 disulfide bonds regenerating a compact structure. The role of this domain in the function of this specialized class of β -1,3 glucanases is unknown. Whether the putative disulfide bonds are involved in the catalytic activity of the proteins must be verified. The location of the catalytic acid residues (Glu-288 in isoenzyme barley GIIb) at the C-terminus near the long extension may suggest a function of the extension in the catalytic activity. In class I and class II chitinases the conserved location of disulfide bounds near the catalytic domain strongly suggests that this region is important for formation of the active site (Araki *et al.*, 1996). Among members of this family, other divergent features are evident: molecular masses of the proteins range from 34 kDa (rice GNS9) to 53 kDa (AraA6); pIs from 6 (wheat Gluc A1) to 9.2 (AraA6). The subcellular localization of members of this subfamily is not known. The phylogenetic proximity to the yeast and bacterial β -1,3-glucanases seems consistent with a long evolutionary history for the class D β -1,3-glucanases.

PsGNS2 expression and developmental processes

Many studies have demonstrated that β -1,3-glucanases are distinct in their gene regulation (Xu *et al.*, 1992; Cabello *et al.*, 1994; Garcia-Garcia *et al.*, 1994; Romero *et al.*, 1998) and isoenzyme localization (Simmons, 1994). The PsGNS2 gene is not induced by defence-related treatment (wounding, salicylate, AlCl_3 , water and *Fusarium solani* inoculation) in leaves and seedling stems and roots. In addition, other genes of class D, namely oilseed rape/*Arabidopsis* A6, olive (Huecas *et al.*, 2001) and rice GNS9 are not defence-related. In contrast, the wheat A1 gene, which is closely related to PsGNS2, is induced under alu-

minium toxicity in roots of seedlings and no transcript is detectable under normal conditions (Cruz-Ortega *et al.*, 1995).

PsGNS2 is weakly expressed in the root and in the shoot of pea seedlings. Furthermore, in different flowering and seed tissues of pea plants, the PsGNS2 transcript was detectable in young flower buds before fertilization and in high levels in the seed coat of pea seeds. The activity of β -1,3-glucanases in pea seedlings has been thoroughly documented. Wong and MacLachlan (1979) purified and characterized two β -1,3-glucanases from stems of pea seedlings grown in darkness.

The detailed analysis of the expression of PsGNS2 in young flower buds led to the detection of the transcript in all four flower whorls, sepals, petals, stamens and carpels including ovules (Figure 2B). In tobacco, class II β -1,3-glucanases are expressed in sepals during flowering and no transcript was found in stamens, petals, placenta, pericarp or developing seeds. Similar but distinct β -1,3-glucanases are expressed during anthesis in sepals, the stigma/style and in the ovary (Côté *et al.*, 1991). In contrast to PsGNS2, all these β -1,3-glucanases are also expressed in leaves in response to pathogens. The function of β -1,3-glucanases in floral tissues is well understood in anthers, where they play an important role in the process of microsporogenesis (Stieglitz, 1977). For example, the oilseed rape β -1,3-glucanase A6 gene is tapetum-specific and temporally expressed. There is a peak in activity when the plant normally expresses callase (a complex of endo- and exo- β -1,3-glucanase activities) that is responsible for the tetrad dissolution (Stieglitz, 1977; Hird *et al.*, 1993). The expression pattern in young flower buds in both PsGNS2 (this paper) and the oilseed rape/*Arabidopsis* A6 genes (Hird *et al.*, 1993) suggest that they have a similar function during flower development in pea. The strong expression of PsGNS2 in the stamens of young flower buds before fertilization indicates a possible role in pollen development and maturation. The expression of PsGNS2 in other floral tissues implies additional functions in the development of floral organs. During both microsporogenesis and macrosporogenesis, all floral tissues undergo rapid cell expansion, and it is possible that β -1,3-glucanases may be involved in cell wall loosening to facilitate cell elongation.

The occurrence of β -1,3-glucanase in relation to fruit development has only been described in pea pods in response to treatment with pathogens or known

elicitors of phytoalexins and ethylene (Mauch *et al.*, 1984).

The present paper constitutes the first report of β -1,3-glucanase expression in relation to seed development. In pea seeds strong expression of *PsGNS2* was visible in the seed coat. The mRNA pattern during seed coat development indicated a temporally regulated gene expression of *PsGNS2*. A strong *PsGNS2* mRNA accumulation in the seed coat corresponded, for the genotype we used, to the phase of enlargement of the seed coat, which ends when the cotyledons reach the contact point with the seed coat. The seed coat is more than a protective organ because of its important role in the nutrition of the embryo. In the pea seed coat, all phloem unloaded assimilates have to pass symplastically the thin-walled parenchyma before they are released into the apoplast and taken up by the embryo (Patrick, 1990). The seed coat could influence embryo development through assimilate supply, which can affect the cell division phase in wheat endosperm (Singh and Jenner, 1984). In legumes, the seed coat modulates nutrient supply to the embryo as has been shown for carbohydrates and nitrogen (Rochat and Boutin, 1992; Weber *et al.*, 1998). Furthermore, temporary starch accumulation is visible in the inner thin-walled parenchyma. In addition to the temporally regulation during development, the expression of *PsGNS2* in the seed coat is also spatially regulated. The transcript is only detectable in the thin-walled parenchyma of the seed coat (Figure 3). This very localized expression of *PsGNS2* implies a specific role of this β -1,3-glucanase in the functioning of the thin walled parenchyma during seed coat development. The thin-walled parenchyma has to guarantee the highly regulated movement of assimilates to the embryo. The pathway of photosynthate transfer has been extensively studied in *Vicia faba* (Offler and Patrick, 1993) and to a lesser extent in pea (Rochat *et al.*, 1996; Tegeder *et al.*, 1999). An extensive lateral transfer of assimilates occurred via plasmodesmata in non-vascular regions of the seed coat. Plasmodesmata are reported to be grouped in pit-fields in the *V. faba* seed coat and many of them exhibited callose deposition in their neck region (Offler and Patrick, 1993). We detected numerous pit-fields of plasmodesmata also in the pea seed coat (Figure 3). Indeed, this study pointed out that the zone in which *PsGNS2* transcript was abundant showed less callose deposits than the other ones. The function of the developmentally and spatially regulated expression of a β -1,3-glucanase in the thin-walled parenchyma of the seed coat could be for

maintaining routes for lateral transport and cell-cell communication through plasmodesmata possibly via the callose hydrolysis. This observation supports the hypothesis that *PsGNS2* plays a significant role in the physiological function of the thin-walled parenchyma as a storage and transfer tissue. Similarly, the expression of a β -1,3-glucanase gene is induced during the early somatic embryogenesis of *Cichorium*; when its transcript becomes abundant, the amount of callose deposited in cell wall surrounding the embryogenic cells decreases (Helleboid *et al.*, 2000). A similar function is suggested for the β -1,3-glucanases in seedling stems, where callose-like material is detected in and near wall pit-fields of parenchymal tissue (Wong and MacLachlan, 1980).

Our results suggest that *PsGNS2* may have a primary function in plant growth and development in seedlings and in young flower buds where *PsGNS2* may be involved in pollen development and maturation. Furthermore, this report of expression of β -1,3-glucanase in the seed coat suggests a specific role of these enzymes in the seed coat during seed development. On the other hand, *PsGNS2* may be expressed in a pre-emptive fashion to protect tissue that are particularly vulnerable to pathogen attack. Additional work is required to understand the function.

PsGNS2-promoter analysis

The abundance of *PsGNS2* transcript in the seed coat led us to assess the specificity of expression of the promoter in a heterologous system in order to establish the generic nature of this observation. We chose the model legume plant *Medicago truncatula*, which is phylogenetically close to pea (Doyle, 1998). This study, with 4 independent transgenic *PsGNS2*-promoter-GUS lines, confirmed the seed specificity of this gene. GUS activity measurements allowed us to detect a background expression in young seedlings and roots and no expression at all in other vegetative tissues (Figure 6). Moreover, expression in flowers was not detectable in fluorometric measurements.

In the seed, GUS activity was very low in the embryo and most of the expression was therefore confined to the seed coat and, to a stronger extent, to the cellular endosperm (Figure 6A and B). The triploid nature of this tissue was confirmed by flow cytometry analysis (C. Mousset-Declas, INRA, Dijon, as described in Lemontey *et al.*, 2000). *In vitro* GUS staining of entire developing seeds, and of their dissected parts, confirmed the GUS activity measurements. This

showed that the strongest GUS expression occurred in the endosperm that appears as a discrete stained blue gelatinous tissue in young seed (Figure 7B, C, F). GUS gene expression in the seed coat of *M. truncatula* was restricted to a small part of the seed coat situated next to the radicle sheath (Figure 7B, 7E). By comparison, no coloration was visible in the embryo (Figure 7D).

The assessment of the β -1,3-glucanase promoter in a heterologous system has confirmed the seed specificity of this promoter and, more precisely, its ability to govern the expression of the GUS gene in the tissue surrounding the embryo but not in the embryo. However, we found some disparities of expression in the *Medicago* background. The main finding was the strong GUS expression driven by the glucanase promoter in the endosperm of the *M. truncatula* seed. Such unexpected observation may contribute further to our understanding of seed development. A possible explanation of this result resides in the observation that the development of endosperm is very different in importance in pea seed and in *M. truncatula* seed. In pea seed, the endosperm appears as an ephemeral coenocytic layer surrounding a large vacuole and the embryo before maturation (Marinos, 1970). Conversely, in *M. truncatula* seed, endosperm development is far more important and it constitutes a discrete tissue (Figure 7) which remains during maturation. The comparison of the expression of β -1,3-glucanase in the pea seed and of the glucanase promoter-GUS construct in *M. truncatula* seed suggests that some parts of the seed coat and the endosperm might have common functional properties. Indeed, endospermic leguminous seed such as fenugreek or *Medicago sativa* contain galactomannan polysaccharides as a substrate reserve in their endosperm (Reid and Bewley, 1979). These polysaccharides are usually ascribed a storage role analogous to starch in other non-endospermic leguminous seeds. Other explanations include the possibility that β -1,3-glucanase might be associated with the release of oligosaccharides which are important for embryogenesis (Berger, 1999). Such a role has been suggested recently for an endochitinase specifically expressed in carrot seed integument (van Hengel *et al.*, 1998). Finally, both seed coat thin-walled parenchyma and endosperm layers are tissues subjected to partial degradation during development (Weber *et al.*, 1995; Berger, 1999). These apoptotic processes may also be related to glucanases.

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