

Original article

Molecular analysis of flax 2S storage protein conlinin and seed specific activity of its promoter

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Abstract

The biochemical properties of conlinin, a 2S storage protein of flax (*Linum usitatissimum* L.), are known but the gene encoding the protein has not been defined. Here, we report the identification of cDNA and genomic clones of two conlinin genes, *conlinin1* (*cnl1*) and *conlinin2* (*cnl2*). The cDNAs contain a single exon encoding a polypeptide containing a putative signal peptide and some features (conserved cysteine residues, glutamine-rich stretches, amino acid composition) characteristic of 2S storage proteins. The promoter regions of the two conlinin genes contain G-box and RY-like cis-acting elements. Northern analysis indicated that the genes are preferentially expressed in developing seeds with the maximal level at 20–35 d after flowering. Analysis of transgenic flax and *Arabidopsis thaliana* containing the GUS reporter gene under the control of the *cnl1* promoter confirmed the seed specific pattern of expression.

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Keywords: Albumin; Conlinin; *Linum usitatissimum*; Promoter; Seed specific expression

1. Introduction

Embryo storage compounds play an important role in the survival of a young seedling by providing the source of carbon and nitrogen skeletons. Embryo storage proteins represent a significant part of the human diet as well as animal feed, and consequently, they have been intensively studied.

In linseed flax (*Linum usitatissimum* L.), the storage protein content ranges from 10% to 37% of the seeds [25]. The major protein fraction, which corresponds to 12S storage proteins, was named linin, and the low-molecular-mass protein fraction (2S) was named *conlinin* [28]. Their contents were reported as 20–42% for conlinin and 58–66% for linin [18,19,30]. Conlinin, isolated by Madhusudhan and Singh [18], was characterised by a sedimentation coefficient of 1.6S, a single polypeptide chain with molecular mass of 15–17 kDa with a more ordered structure when compared to the 12S fraction and stability over a wide range of pH and

temperature. No trypsin inhibitor or protease activity sometimes associated with small molecular weight seed proteins was observed. Although the amino acid composition of conlinin has been studied and published previously [18,30], no sequence data are available for the genes.

The regulatory sequences of the storage proteins are probably the most studied class of promoters. One of the reasons is the broad spectrum of genes available, as the storage proteins are often present in large gene families differing in their expression patterns. Another reason is the apparent practical utility of seed-specific promoters in crop improvement, production of valuable proteins or modification of seed storage compounds.

Linseed flax is an important oilseed crop and as such constitutes an obvious target for genetic engineering efforts to improve its agronomic performance, modify oil composition and as a suitable vehicle crop for biofarming [22,23,27]. Techniques facilitating an introduction of new genes into flax are several and have been known for a relatively long time [15,21,29]. Another essential element in genetic engineering projects is promoters that can drive the expression of genes of interest in a desired tissue specific manner and at sufficiently high levels.

Up to now, only two homologous flax promoter sequences have been described in the literature, both being the upstream

Abbreviations: *cnl1*, *cnl2*, genes for flax 2S storage protein *conlinin1* and *conlinin2*; CaMV, cauliflower mosaic virus; DAF, days after flowering.

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regions of stearoyl-acyl carrier protein desaturase (SAD) genes [14]. The expression of the *sad2* promoter is relatively strong and constitutive, whilst the *sad1* promoter is characterised by seed specific, but low expression.

In this report, we describe the identification of two flax conlinin cDNAs *cnl1* and *cnl2* and their genomic sequences. The *cnl1* encodes a 168 amino acid-long polypeptide with a molecular mass of 19 kDa and is specifically expressed at the middle-late stage of seed development (10–35 d after flowering (DAF)). Several cis-elements, previously identified as critical for seed specific expression of albumin genes, were found in the promoter regions of conlinin genomic sequences. Analysis of the *cnl1* promoter in a fusion with GUS in transgenic flax plants demonstrated that the promoter is specifically activated in the developing seeds including the embryo and the inner layer of the seed coat. Analysis of the same construct in *Arabidopsis thaliana* indicated the specific expression in the developing embryo.

2. Results

2.1. Identification of cDNAs encoding flax 2S albumin-conlinin

Based on the dot-blot experiments (see Section 4.3), the cDNAs preferentially expressed in the developing flax seeds can be divided into three groups. The first group (53.5% of

analysed clones) has similarity to 2S storage proteins and the second group (9.4%) to 12S storage proteins from other plant species. The third group (37.1%) consists of cDNAs not hybridising with either group.

Two clones (*cnl1* and *cnl2*) from the first group were fully sequenced and studied. The *cnl1* (EMBL accession number AJ414732) is 673 bp long with the longest ORF coding for 169 amino acids with molecular mass 19 kDa and predicted isoelectric point 7.5. The *cnl2* (AJ414733), probably encoded by another member of the gene family, is 676 bp long and codes for 168 amino acids. The difference in nucleotide sequences between *cnl1* and *cnl2* is relatively small, comprising 43 point mutations and one 3 base-long deletion within the predicted open reading frame of *cnl2*. Additional differences are present in the 5' and especially 3' untranslated regions. The deduced conlinin proteins do not possess a significant homology to the sequences in databases when analysed by *blastx* and *blastp* searches; however, short stretches surrounding structurally conserved cysteine residues are aligned with 2S storage proteins of species such as *Gossypium hirsutum*, *Brassica napus* and *Arabidopsis thaliana* (Fig. 1). A comparison of the two protein sequences indicated an 88% amino acid identity throughout the sequences. The positions of cysteine and most of the glutamine residues, typical for 2S albumins, are all conserved between the two proteins (Fig. 1). Computer prediction of subcellular targeting by the TargetP programme

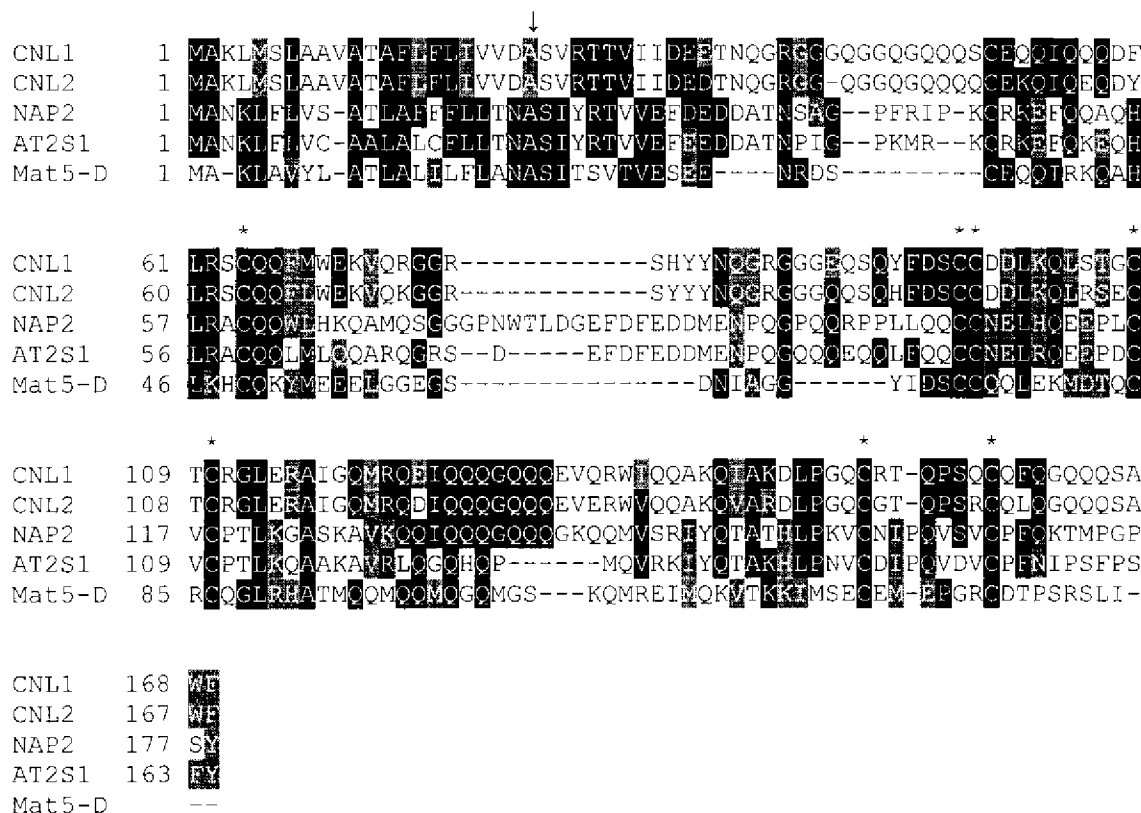


Fig. 1. Comparison of deduced conlinins and some known 2S storage proteins. *cnl1*, *cnl2*, conlinins; NapB, *B. napus* (S07828); At2S1, *A. thaliana* (P15457); Mat5-D, *G. hirsutum* (T09850). The conserved cysteine residues are marked by asterisks; the arrow marks the end of predicted signal peptide.

Table 1

Comparison of previously published data on conlinin and the predicted proteins. A, molecular mass; B, amino acid composition of small-molecular-mass fractions of flax seed proteins as described previously and deduced conlinins after cleavage of the putative signal peptides

	Literature	Predicted proprotein		
	Madhusudhan and Singh [18]	Youle and Huang [30]	CNL1	CNL2
A. Mol. mass	15 000–17 000	–	16 769	16 718
B. (mol.%)				
Ala	1.9	5.1	2.7	2.7
Asx	13.1	6.0	5.4	6.8
Cys	3.5	8.2	5.4	5.5
Glx	35.0	23.8	29.9	30.1
Phe	2.4	2.2	3.4	2.1
Gly	8.3	13.8	12.9	12.3
His	1.6	1.2	0.7	0.7
Ile	2.8	2.9	4.8	3.4
Lys	4.9	6.0	2.7	3.4
Leu	5.4	5.3	3.4	4.8
Met	0.8	1.9	1.4	0.7
Pro	3.0	1.6	1.4	1.4
Arg	13.1	6.0	7.5	8.2
Ser	3.9	6.1	6.1	5.5
Thr	2.1	3.6	4.1	3.4
Val	2.6	3.9	2.7	4.1
Trp	2.0	0.8	2.0	2.0
Tyr	1.4	1.5	2.0	2.7

[8,24] suggests the presence of a 22 amino acid-long signal peptide targeting both protein precursors into the secretory pathway (score 0.993 and 0.923; reliability class 1).

In flax, there is no molecular sequence information available for seed storage proteins. The published data on flax storage protein linin (12S) and conlinin (2S) are limited to biochemical analysis of the protein size and amino acid contents [18,30]. Analyses of the sequence-deduced polypeptides revealed that the amino acid composition and size (after the cleavage of the putative signal peptide) of the proproteins encoded by the clones *cnl1* and *cnl2* are very close to that of the flax conlinin published previously (Table 1). Considering the fact that the biochemical analyses of amino acids reflect the mixture of mature proteins encoded by different members of the gene family (at least four members according to our Southern blot analysis, data not shown), we believe that *cnl1* and *cnl2* are members of the gene family coding for conlinin storage protein in flax.

2.2. Northern blot analysis of the conlinin expression

To precisely define the expression pattern of conlinin genes, two northern blots with total RNA isolated from hypocotyls, leaves, roots, stem, flower buds and embryos in different developmental stages from field grown flax plants were hybridised with the *cnl1* cDNA probe. In terms of tissue specificity, a single strong band was detected only in the developing embryos, even after a prolonged exposure (Fig. 2A). In the embryos, the hybridisation signal was first

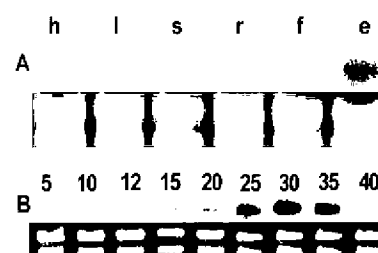


Fig. 2. Northern blot analyses of conlinin expression in different tissues (A) and the temporal pattern of expression in flax embryos (B). The lower part of each pane illustrates the amount of total RNA loaded; h, hypocotyl; l, leaves; s, stem; r, roots; f, flower buds; e, embryos; 5–40, DAF.

detected at about 10 DAF and the expression then gradually increased reaching the maximal level at 25–30 DAF (Fig. 2B).

2.3. Identification of flax conlinin promoters

Considering the expression pattern of conlinin and the previously proven utility of 2S albumin promoters in other species, the cDNA clone *cnl1* was used to screen the flax genomic library in an effort to clone the respective promoter region. Four genomic clones have been identified. Two provide the same band pattern upon restriction enzyme digestion (data not shown) and the coding region is identical to the *cnl1* cDNA clone. The other two clones contain the *cnl2* coding region. In the vicinity of *cnl1*, 932 bp downstream from its stop codon, another sequence bearing similarity to *cnl1* and *cnl2* was found. This, however, is probably a pseudogene with a stop codon located only 117 bp from the start of the open reading frame.

In the upstream region from the predicted start codon, several cis-elements previously identified as crucial for seed specific expression of the napin gene were found in *cnl1* and *cnl2* promoters. An RY (CATGCA) element is located 5 bp downstream from the typical G-box (CACGTG). In addition, there is another copy of a slightly modified G-box (TACGTG) containing the core ACGT motif and also a modified RY (CATGAA) overlapping a perfect RY on the opposite strand located 16 bp upstream from the first G-box (Fig. 3). The *cnl2* promoter contains a similar organisation of cis-acting sequences with the exception of one base change in the distal RY element.

2.4. Expression of GUS reporter gene under control of *cnl1* promoter in transgenic flax and *A. thaliana* plants

To study the expression pattern of a conlinin promoter, the *cnl1* 1 kb-long promoter region was placed in front of the uidA (GUS) reporter gene in a binary transformation vector and the construct was used to transform flax and *A. thaliana* plants. When the transgenic flax plants were analysed histochemically, no GUS activity was detected in the roots, stem, leaves or floral buds. In the seed, the reporter gene was expressed throughout the embryo as well as the seed coat. The GUS activity present in the seed coat, however, segre-

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cnl1  tgCATGAAtaatacTACGTGtaagcccaaaagaaccCACGTGtagccCATGCAaagtt
cnl2  tgCATGTATAatacTACGTGtaagctcagaaaattcCACGTGtcgccCATGCAaaatt
napA  cacacacatacaatcacatgcgtgCATGCAattattaCACGTGatcgcCATGCAaatct

cnl1  aacactcacgacccattcctcagtcctccacTATATAAA
cnl2  aacactcacgaccc-attcctaaatctccacTATATAAA
napA  cctttatagccTATATAAA

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Fig. 3. A portion of *cnl1* and *cnl2* promoters containing TATA-box, RY (CATGCA), G-box (CACGTG) cis-acting elements (in capitals) as well as their modified versions (TACGTG) and (CATGAA), respectively. For comparison, the corresponding region of napin promoter (J02798) is depicted.

gated together with the activity within the embryo (Fig. 4A) implying that GUS staining in the seed coat may be the result of the expression in the residual layers of endosperm cells [6,9] rather than integuments or any other maternal tissue. Within the embryo, blue staining was more intense in the embryo axis than the cotyledons (Fig. 4B). This, however, could also be caused by easier access of the enzymatic substrate and more intensive staining of vascular tissue as observed previously in other species [20].

Four transgenic flax plants containing a single copy of a GUS reporter gene under the control of *cnl1* promoter (Southern blot not shown) were analysed quantitatively by fluorescent assay (Fig. 5). Compared to two reference plants, single-copy transgenic flax transformed by pBI121, plants containing the *cnl1* promoter-GUS fusion possessed, in most cases, considerably higher GUS activity in their developing seeds at 20 DAF. The enzyme activity in other assayed plant organs (roots, stems and leaves) did not exceed the background.

To establish the contribution of the seed coat to overall seed expression, seeds of one transgenic line (*cnl-9*) were analysed as dissected embryo and seed coats at 15, 20 and 25 DAF. The results show that the seed coat contains a considerable portion of total seed GUS activity at day 15 (66.5%), but its share diminishes as the activity in the embryo increases with time, and represents 34.9% at 20 and 26.6% at 25 DAF.

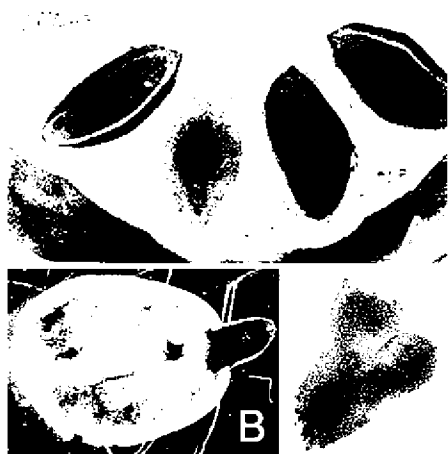


Fig. 4. Histochemical staining of GUS activity in transgenic flax and *A. thaliana*. A, transverse section of flax capsule; B, extracted flax embryo; C, extracted embryo of *A. thaliana*.

To assess the distribution of promoter activity within the embryo, *cnl-9* embryos were dissected into two parts — one containing only one cotyledon and the other containing the rest of the embryo including the embryo axis (see Section 4.9). The results showed that stronger histochemical staining in the embryo axis is not artefactual as this relatively small portion of the embryo mass contained about 30% of the total GUS activity.

Similar to the expression pattern in flax, *A. thaliana* T1 seeds that were detected as transformed by direct histological staining of the developing seeds within the siliques, blue staining was spread throughout the embryo including cotyledons, although somewhat more intense in the embryo axis (Fig. 4C). No staining was observed in other parts of the seed.

3. Discussion

Conlinin, as a small molecular fraction of flax seed proteins, was first described by Vassel and Nesbitt [28]. Since then, several biochemical analyses of the small-molecular-mass flax proteins were reported [18,30]. But up to now, these proteins have not been characterised at the molecular level. We applied differential screening of the embryo specific cDNA library and cloned the conlinin gene. The abundance of isolated clones in the cDNA library, the high level

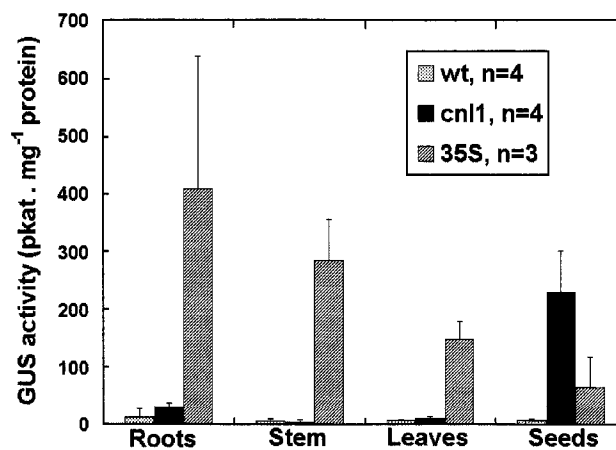


Fig. 5. GUS activity in seeds and vegetative organs. GUS activity was measured by fluorometric assay. Wt, wild type; *cnl1*, conlinin1 promoter; 35S, CaMV 35S promoter; *n*, number of independent plants or transgenic lines analysed; error bars, standard deviation. The sample for each assay consisted of 20 T1 seeds from at least three capsules 20 DAF or, in the case of vegetative organs, 20 T1 plants.

and seed specificity of the expression and temporal pattern of the transcription, as well as the presence of a signal peptide, conserved glutamine-rich stretches and cysteine residues, and the overall amino acid composition of the predicted proteins strongly suggest that these cDNAs and their genomic counterparts indeed encode conlinin.

We have also analysed the spatial and temporal expression pattern of the *cnl1* promoter in the fusion with GUS reporter gene in transgenic flax plants. These experiments confirmed the seed specific expression as well as the temporal pattern typical for seed storage proteins. We also observed an unexpectedly high level of expression in the inner layers of the seed coat, especially at the early stages of seed development. Given that the GUS staining in the seed coat is co-segregating with the reporter expression in the embryo, we believe that the detected GUS activity is limited to the residual endosperm layer without involvement of maternal tissues. The expression of napin and cruciferin genes in *B. napus* endosperm was reported by Höglund et al. [13]. However, the level of accumulation of both proteins in the endosperm was considerably lower than that in the embryo and the immunohistochemical analyses detected their presence in the endosperm only after the onset of embryo accumulation. Our results correspond more to the model of a transient accumulation of storage proteins in the endosperm of *Vicia faba*, in which the immunohistochemical analyses of vicilin and legumin deposition in developing seeds showed involvement of both endosperm and suspensor in a transient synthesis and accumulation of these proteins at precisely defined stages [26]. These storage compounds are, however, rapidly metabolised by the growing embryo. Panitz et al. [26] also suggested that storage protein expression is post-transcriptionally regulated in parts of the endosperm as there is a significant difference between the patterns of mRNA and protein accumulation. However, neither metabolic degradation nor post-transcriptional regulation would be expected to be reflected in an experiment where the product of a chimaeric gene expression is a protein as stable as GUS. As a consequence, the biological importance of the endospermic conlinin expression in the later stages of flax seed development is probably less than what might appear from our results with the GUS fusion.

The promoter regions of both conlinin promoters contain some of the regulatory elements identified to confer high levels of seed-specific expression in other species. Specifically, RY and G-box elements identified in the napin and other promoters as essential for its seed-specific function are also present in the *cnl1* and *cnl2* promoters. In the napin as well as in *A. thaliana* 2S albumin genes, the G-box is surrounded by two RY elements (RY/G/R) [10]. The conlinin promoters, in contrast, have only one RY element (G/R) (Fig. 3). Another pair of modified G-box and RY elements is located 16 bp upstream from the first G-box. In the napin promoter, in addition to the RY/G cluster, an ABA-responsive element B-box located further upstream was identified

as essential for its regulation [10]. No similar motif could be identified in the conlinin promoters.

B. napus and *A. thaliana* 2S storage proteins are encoded by small gene families [16,17], members of which are differentially expressed throughout the embryo [2,12]. Using the GUS fusion, we have observed a preferential expression of *cnl1* in the embryo axis of transgenic flax seeds and, although to a somewhat lesser degree, in the transformed seeds of *A. thaliana*. In flax, the distribution of GUS activity and the preferential expression in the embryo axis was also confirmed by a quantitative assay. A similar pattern of expression has been described for the *At2S1* gene encoding 2S albumin of *A. thaliana* [12]. Conceição da Silva and Krebbers [5], based on their experiments with the hybrid promoters of the *Arabidopsis* 2S gene family, indicated involvement of the CATGCA motif located upstream of the RY/G group in the differential expression of *At2S1* and *At2S2* genes in the cotyledons of *A. thaliana*. The conlinin promoter contains, in addition to the CATGCA adjacent to the G-box, another modified motif (CATGAA) in a "+" orientation overlapping a perfect CATGCA in a "-" orientation. It would be interesting to determine whether these overlapping motifs are involved in the spatial regulation of the conlinin genes, particularly in view of the presence of a single base difference between *cnl1* and *cnl2* promoters (CATGAA and CATGTA, respectively) amid highly conserved region.

4. Methods

4.1. Plant material

Field grown plants of linseed flax (*Linum usitatissimum* cv CDC Normandy) were used as a source of wild type material. Transgenic plants were grown in a growth chamber under continuous fluorescent illumination ($150\text{--}200\ \mu\text{E m}^{-2}\text{ s}^{-1}$) at $22 \pm 2\ ^\circ\text{C}$. Developing seeds were harvested at specified dates after the individual flowers were labelled at the day of their full opening. Fifteen-day old seedlings of the same variety were used for the isolation of flax genomic DNA used in Southern analyses and genomic library construction.

4.2. Recombinant DNA methods and sequencing

Nucleic acid preparations, manipulations, and construction of cDNA and genomic libraries were done following instructions of manufacturers or according to standard protocols [1]. DNA sequencing and oligo-syntheses were performed at the Plant Biotechnology Institute in Saskatoon, Canada.

4.3. Embryo-specific cDNA library preparation and screening

A cDNA library was prepared from flax embryos dissected from developing seeds at 20 DAF in the Uni-Zap XR

EcoRI and XhoI predigested lambda vector (Stratagene, La Jolla, USA). The embryo-specific cDNA clones were then identified by differential screening. Pairs of identical phage plaque lifts containing DNA of approximately 6×10^4 clones were hybridised with double stranded ^{32}P -labelled cDNA probes derived from seedlings and 20-d-old embryos, respectively. Isolated embryo-unique clones were ordered on microplates and used as templates in PCR amplification of their respective inserts using vector specific T3 and T7 primers. Five hundred and seventy-six PCR products were arrayed on a positively charged nylon membrane. Membranes were hybridised with biotin-labelled probes prepared by chemical labelling (Boehringer-Mannheim, Germany) of randomly chosen amplicons. The cDNA clones were classified based on their hybridisation with the probes.

4.4. Southern blotting

Flax genomic DNA was digested by BamHI, EcoRI and HindIII, blotted onto the nylon membrane and processed according to standard protocols [1]. The ^{32}P -labelled cDNA clone *cnl2* was used as a probe.

4.5. Flax genomic library preparation and screening

High-molecular-mass genomic DNA was prepared from 15-d-old seedlings using the CTAB procedure combined with Qiagen Genomic Tip (Qiagen, Hilden, Germany) purification [7]. Genomic DNA was partially digested with MboI, fractionated on a sucrose gradient and cloned into Lambda FixII XhoI predigested vector (Stratagene, La Jolla, USA). Approximately 8×10^5 clones were screened using a ^{32}P -labelled cDNA probe representing the putative conlinin transcript. The upstream regions were sequenced using cDNA internal primers.

4.6. Cloning of *cnl1* promoter region and construction of binary vector

The promoter region of the *cnl1* gene was amplified from an isolated genomic clone using the recombinant thermostable DNA polymerase DyNAzyme EXT (Finzymes, Espoo, Finland). The HindIII and XbaI sites were added to the 5'-ends, respectively, to facilitate further cloning. The 1058 bp PCR product was first cloned into pCR2.1 using the TOPO-TA cloning system (Invitrogen, Carlsbad, USA) and the promoter was then excised and cloned into HindIII/XbaI sites of pBI121 binary vector (Clontech, Palo Alto, USA), replacing the original cauliflower mosaic virus (CaMV) 35S promoter and resulting in pBI-*cnl1*-GUS.

4.7. Flax and *A. thaliana* transformation

Agrobacterium tumefaciens strain GV3101(pMP90) harbouring pBI-*cnl1*-GUS was used to transform flax hypocotyls essentially as described by Jordan and McHughen [15], except that epidermis was not removed from hypocotyl seg-

ments. Control plants were transformed by pBI121 containing the original CaMV 35S promoter. Transgenic shoots were selected by means of the regeneration assay as described in Jordan and McHughen [15]. *A. thaliana* plants were transformed by infiltration of *A. tumefaciens* carrying the pBI-*cnl1*-GUS binary vector as described in [4].

4.8. Histochemical GUS staining

Hand sectioned capsules and individual seeds with or without the seedcoat were fixed for 3 min in 5% glutaraldehyde and stained overnight in the staining solution according to Fütterer et al. [11]. Alternatively, tissues were stained directly and fixed after staining by formaldehyde/acetic acid/ethanol/water (1/1/4/14, v/v/v/v).

4.9. Fluorimetric GUS assay

Four single-copy transgenic flax plants were selected based on their segregation ratios and the number of T-DNA inserts was confirmed by Southern blotting. Twenty developing seeds at 20 DAF from these plants, as well as from two single-copy control plants transformed by pBI121, were combined and used for the analysis of GUS activity. Seeds were ground in 3 ml extraction buffer [11] by polytron. The volume of the extract was increased to 12 ml and then samples were centrifuged at $12\,000 \times g$ for 30 min at 4 °C. The supernatant was extracted with two volumes of hexane to facilitate later use of the Bradford protein assay [3] and 2 µl of the aqueous fraction were used in the assay of GUS activity essentially as described in [11].

To assess the distribution of GUS activity between the cotyledons and the embryo axis, one cotyledon was removed from the embryo and assayed separately along with the rest of the embryo including the axis. Three embryos from *cnl-9* plant 20 DAF were assayed individually and the results were expressed as percentage of the total GUS activity per embryo.

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