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MOLECULAR CHARACTERIZATION OF THE *LINUSITIN*-LIKE GENE FAMILY FROM FLAX

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Osmotin-like proteins from the fifth class of pathogenesis-related proteins (PR-5), including linusitin that we isolated from flax seeds, have been characterized in many plant species. A cDNA library has been constructed from flax (*Linum usitatissimum* L.) seedlings to analyze the osmotin-like gene family from flax. A cDNA *LIN1* was isolated from the library and found to exhibit significant similarities to genes encoding osmotin-like proteins and thaumatin from *Thaumatococcus daniellii*. The deduced LIN1 protein contains all 16 cysteine residues that are conserved in all PR-5 proteins as well as other residues important for antifungal activity. The *LIN1* cDNA encodes a preprotein, which is subsequently processed into the mature protein by removal of an N-terminal signal peptide. Southern blot analysis showed that the *LIN1* gene family is encoded by few copies in the flax genome. The gene encoding LIN1 is intronless and developmentally regulated, and transcripts accumulate in an organ-specific manner in healthy flax seedlings. The *LIN1* mRNA was constitutively expressed, predominantly in root tissue. The comparison of N-terminus sequences from linusitin-like protein (LIN1) and previously isolated linusitin confirmed differences in amino acid composition and demonstrated the occurrence of at least two osmotin-like proteins in flax.

Keywords: osmotins, PR-5 proteins, linusitin, flax, gene expression.

Introduction

One of the ways in which plants respond to biotic and/or abiotic stress is the accumulation of pathogenesis-related proteins (PR proteins). They are a large group of low molecular mass proteins, comprising a steadily increasing number of distinct classes of evolutionarily and structurally related proteins (Van Loon and Van Strien 1999). The members of the PR-5 class are evolutionarily conserved in the plant kingdom and have been isolated from different plant species (Anžlovar and Dermastia 2003). Acidic forms of PR-5 proteins are often designated as thaumatin-like proteins because they show a certain similarity to the sweet-tasting protein thaumatin isolated from the fruit of the West African monocot *Thaumatococcus daniellii* (Van der Wel and Loeve 1972). In addition, a basic isoform was originally isolated from salt-adapted tobacco cells and called osmotin. Osmotin and other closely related proteins, usually referred to as osmotin-like proteins, have been characterized in several plant species. Osmotin was shown to accumulate in the vacuole of the cell. In contrast with the apoplastic PR-5 proteins, it contains a C-terminal propeptide that is considered to determine vacuolar targeting (Anžlovar and Dermastia 2003).

Although the physiological function of PR-5 proteins is not yet fully understood, they have been reported to have an antifungal activity in *in vitro* assays (Anžlovar and Dermastia 2003). In addition to the induction of PR-5 proteins following biotic or abiotic stress, they are also constitutively present in many plant tissues (Van Loon and Van Strien 1999; Anžlovar and Dermastia 2003). PR-5 and PR-5-like proteins have been reported to accumulate in healthy plants in specific organs and tissues: in seeds (Cvetkovic et al. 1997; Anžlovar et al. 1998; Shih et al. 2001), in old leaves (Capelli et al. 1997), and during fruit ripening (Fils-Lycaon et al. 1996; Salzman et al. 1998; Hong et al. 2004) and flowering (Cheong et al. 1997). These observations raise the question as to whether *PR* genes evolved primarily to limit damage by invading pathogens or were adapted from other functions to serve an accessory protective role (Van Loon and Van Strien 1999).

Previously, we reported the biochemical characterization of the basic 25-kDa PR-5 protein linusitin from flax (*Linum usitatissimum* L.) seeds, together with a model for its antifungal activity by membrane permeabilization (Anžlovar et al. 1998). In the present study, a new linusitin-like protein was purified from flax radicles. In order to resolve whether this is the same protein or is different from that in seeds, we compared their N-terminal sequences. Moreover, we determined and characterized the full-length cDNA clone sequence for the radicle protein, which is, to our knowledge, the first report of any *PR-5* gene sequence from flax.

Material and Methods

Plants

Seeds of flax (*Linum usitatissimum* L.) were obtained from Semenarna Seed Company. They were germinated in plastic germination chambers (4-d-old seedlings) or in soil (20-d-old plants). Seedlings and plants tissues were harvested and

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immediately frozen in liquid nitrogen and stored at −70°C for RNA and DNA isolation. For linusitin-like protein isolation, radicles of 4-d-old seedlings were harvested and immediately frozen in liquid nitrogen.

Protein Purification and Amino Acid Sequence Analysis

Linusitin-like protein was purified from flax seeds as described previously for linusitin (Anžlovar et al. 1998). Because of the tight binding of protein to the membrane filters, lyophilization was used to replace membrane concentration used previously. The purity of the protein was estimated by SDS-PAGE with PhastSystem (Pharmacia LKB Biotechnology AB). The separating gel contained a gradient of acrylamide from 8% to 25% with 0.5% SDS. Protein bands were visualized by silver staining.

The first five amino acids from the N-terminus of the linusitin-like protein were determined by automated Edman degradation performed on an Applied Biosystems 475 liquid sequencer connected online to a model 120 A phenylthiohydantoin–amino acid analyzer from the same manufacturer. The sequence was compared to the N-terminus of linusitin.

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[1] aattctcatgagctaaagtatccccttaattactaccaaaatcatgacccactttcccatt
                                                (1) M  T  H  F  P  I
    ttgctcatccccttgttttcctccttgctactttgggtctccatcaccaatgcagctgtg
     L  L  I  P  L  F  S  S  L  L  L  W  V  S  I  T  N  A  A  V
    attgacatcttcaacaactgtccttacacagtctgggcagcttccacgcccattggtggc
     I  D  I  F  N  N  C  P  Y  T  V  W  A  A  S  T  P  I  G  G
    ggtcgtcaactcgaccacggacaaacatggaccatctaccctcccgctggaacttcaatg
     G  R  Q  L  D  H  G  Q  T  W  T  I  Y  P  P  A  G  T  S  M
    gcccgcatttggggccgccggaattgcaactttgatggcagcggtaggggttggtgcgag
     A  R  I  W  G  R  R  N  C  N  F  D  G  S  G  R  G  W  C  E
    actggcgattgtggtggggtcctcaactgtcagggttggggtgtcccgccgaacactcta
     T  G  D  C  G  G  V  L  N  C  Q  G  W  G  V  P  P  N  T  L
    gctgagtacgcccttaaccaattctcgaatttggacttctacgacatatcgttggtggac
     A  E  Y  A  L  N  Q  F  S  N  L  D  F  Y  D  I  S  L  V  D
    gggttcaacattcctatgatcttcacccccacggcaaacgtgggttcaggtaactgccag
     G  F  N  I  P  M  I  F  T  P  T  A  N  V  G  S  G  N  C  Q
    agcctaacttgcacggctgacatcaacacacagtgccctggtgagttgcgggccccgggc
     S  L  T  C  T  A  D  I  N  T  Q  C  P  G  E  L  R  A  P  G
    gggtgcaacaacccttgtaccgtgttcaagaccaatgagtattgttgcactcaggggtac
     G  C  N  N  P  C  T  V  F  K  T  N  E  Y  C  C  T  Q  G  Y
    gggacttgcggtccgactggattttcaagattctttaaggataggtgtccgacttcttat
     G  T  C  G  P  T  G  F  S  R  F  F  K  D  R  C  P  T  S  Y
    agttaccctcaggacgatccaagcagtacgttcacttgccccggcggtaccaactataga
     S  Y  P  Q  D  D  P  S  S  T  F  T  C  P  G  G  T  N  Y  R
    gtggtgttttgcccctatggctctactcatcntgacatcgacaccaacaacaacaacaat
     V  V  F  C  P  Y  G  S  T  H  X  D  I  D  T  N  N  N  N  N
    aacaacaagaatgtgacgttggctatggtgaccgagaagatcaattctgagtagacttat
     N  N  K  N  V  T  L  A  M  V  T  E  K  I  N  S  E  *  (263)
    taaaacaatgtaatgttgtttatgaagaaaaagaaggcgattgcttccagctgtatcaat
    gtgtcaaanagttgatgatgaaatgagaataaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
    aa [964]
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Fig. 1 Nucleotide and deduced amino acid sequences from the *LIN1* cDNA. The nucleotide sequence is numbered, starting with the first nucleotide of the insert, and the amino acid sequence numbering starts with the start codon. The amino acid sequence is translated from the open reading frame. The putative signal peptide and C-terminal extension peptide are underlined. A potential polyadenylation site is indicated by double underlining. The asterisk denotes the stop codon.

PCR for cDNA Probe Preparation

Total RNA was isolated from 4-d-old flax seedlings as described in Chomczynski and Sacchi (1987). Degenerate oligonucleotides were used to amplify a partial cDNA fragment by RT-PCR. The degenerate sense oligonucleotide was designed on the basis of the conserved N-terminal amino acid sequence of PR-5 proteins (5′-ACGAATTCTGYCCNTAYACNGTNTGG-3′). An antisense oligonucleotide primer was deduced from a TGDC peptide domain (fig. 3) that is conserved among PR-5 proteins (5′-CTGGGATCCNCCRCARTCNCCNGT-3′) (Cheong et al. 1997). The resulting PCR product was cloned into a PCR-script vector (Invitrogen) and sequenced. The fragment obtained was used as a probe in cDNA library screening and in Northern blot analysis. The deduced protein sequence from amplified 180-bp cDNA fragment showed the greatest similarity to osmotin-like proteins from *Solanum commersonii* (National Center for Biotechnology Information [NCBI] accession no. P50701) and from *Capsicum annuum* (NCBI accession no. CAC34055.2) (83% identity).

Construction of a cDNA Library and Isolation of a Full-Length cDNA from Flax Seedlings

Total RNA and poly(A)$^+$ RNA were isolated from 4-d-old flax seedlings using RNeasy Plant Mini Kit (Qiagen) and a QuickPrep Micro mRNA kit (Amersham Pharmacia Biotech). The poly(A)$^+$ RNA then served as the template for cDNA synthesis and construction of a λ ZAP-cDNA library according to the manufacturer's instructions (Stratagene). The resulting library contained 1.0×10^5 independent clones. Plaques (10^4) were plated out at low density, and duplicate membranes were lifted from the plates. Membranes were prehybridized for 1 h at 42°C in a solution containing 50% formamide, 6X Denhardt's reagent, 0.5% SDS, and denatured salmon sperm DNA. The blots were hybridized in the same solution for 24 h with the ^{35}S-labeled 180-bp PCR fragment. The radioactive cDNA probe was prepared using random primer labeling with a Random Primed DNA Labeling Kit (Roche). Following hybridization (at 42°C), the membranes were washed in the following solutions: 2X SSC (sodium chloride-sodium citrate) for 5 min; 1X SSC, 0.1% SDS for 5 min; and 0.1X SSC, 0.1% SDS at 50°C for 5 min. Membranes were dried overnight. Autoradiography was carried out with x-ray films (X-OMAT AR, Kodak) with intensifying screens at 25°C for 4 d. The single hybridizing plaque was isolated.

The phagemid (pBluescript SK$^-$) of the positive clone was rescued from phage, following the *in vivo* excision protocol recommended by the manufacturer (Stratagene). The cDNA clone was sequenced using a BigDye sequencing reaction kit (Perkin-Elmer) on an ABI-Prism 310 (Applied BioSystems). Analysis of the nucleotide and amino acid sequence data was performed using the BLAST program (Altschul et al. 1997). Amino acid sequences were aligned using the ClustalW program (Jeanmougin et al. 1998) and formatted using BOXSHADE. Molecular mass and isoelectric point (pI) were obtained using the Compute pI/Mw tool (Bjellqvist et al. 1993), and signal peptide was predicted with the signalP program (Nielsen et al. 1997).

Isolation of Genomic DNA and PCR Amplification of DNA Fragments Containing the LIN1 Gene

Genomic DNA was isolated from 4-d-old flax seedlings by the CTAB method (Rogers and Bendich 1985). DNA fragments were amplified by PCR with the primers NO (5′-TCGGCACGAGGAAAGTATCCCC-3′), C4 (5′-CGGTCACCATAGCCAACGTCAC-3′), and C5 (5′-GACACATTGATACAGCTGGAAAGC-3′), which were designed based on the full-length *PR-5*-like cDNA sequence isolated by the cDNA library in this work.

PCR was performed using standard conditions; only the extension time was prolonged to 4 min. The resulting PCR products were cloned into a pGEM-T-Easy Vector (Promega) and sequenced to determine the presence of introns.

Southern Blot Analysis

One or both restriction enzymes, *Eco*R I and *Bam* HI, were used to digest 10 μg of flax genomic DNA. The resulting fragments were separated on a 0.8% agarose gel and blotted onto a nylon Hybond-N+ membrane (Amersham Biosciences) following a protocol outlined in Sambrook et al. (1989). The blot was hybridized overnight at 60°C with

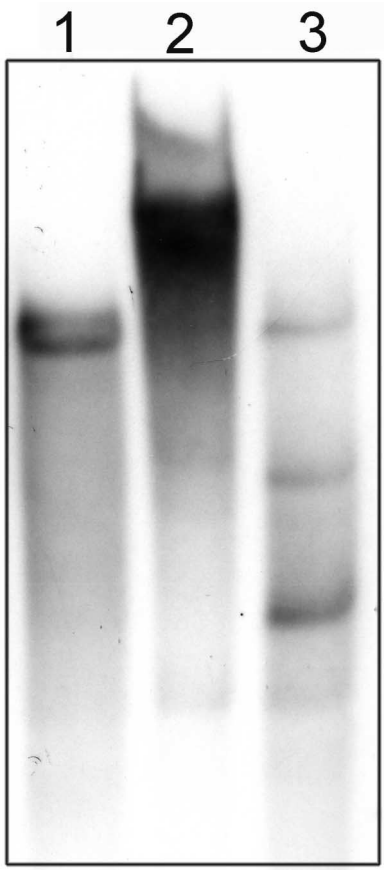

Fig. 2 Southern blot analysis. Genomic DNA was digested with *Eco*R I (*1*), *Bam* HI (*2*), or *Eco*R I and *Bam* HI (*3*). The conditions for DNA fractionation, transfer, hybridization, and washing the membrane are described in "Material and Methods."

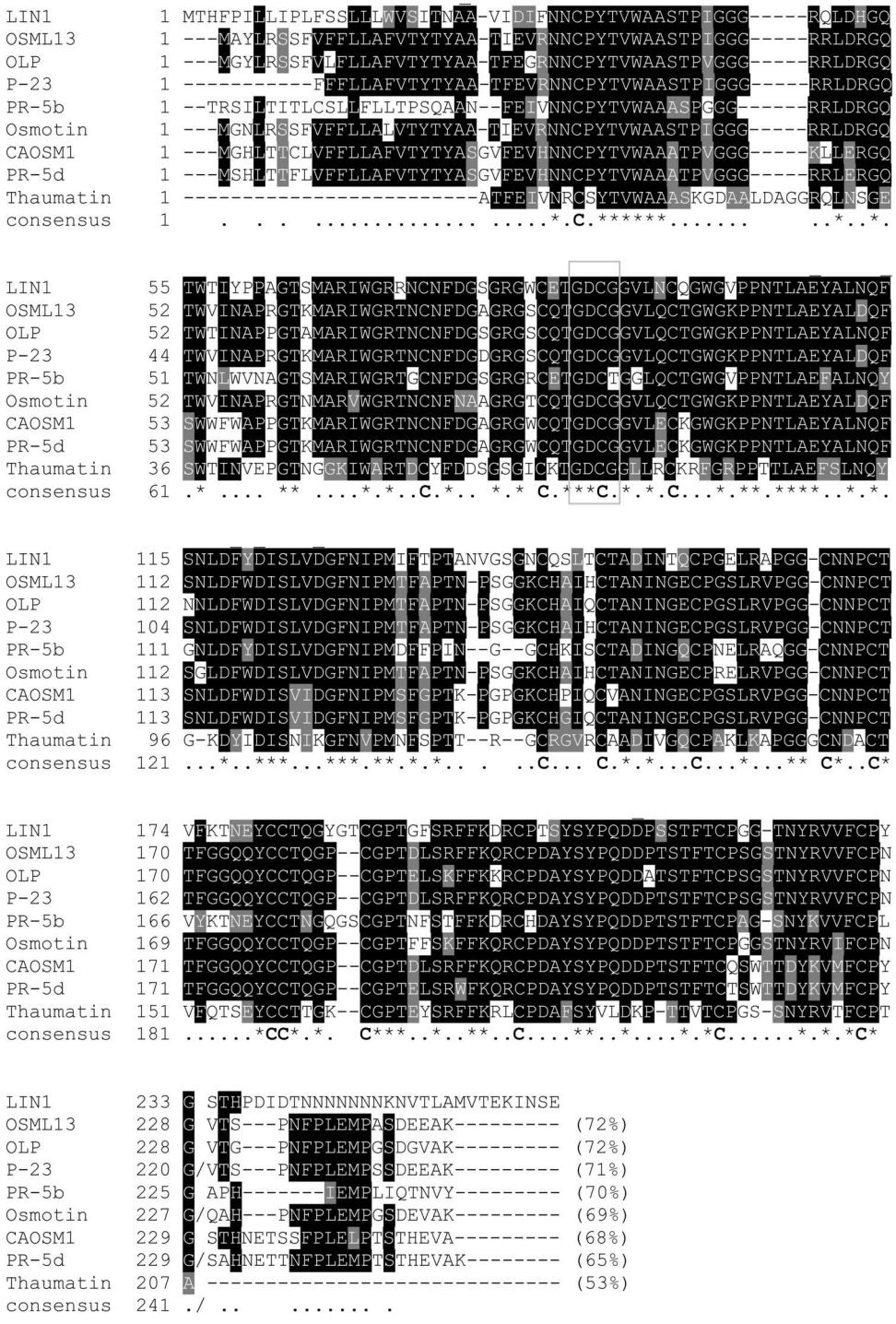

Fig. 3 Comparison of the deduced amino acid sequence of LIN1 with other PR-5 proteins. Identical amino acids are highlighted in black and marked by asterisks, and similar amino acids are highlighted in gray and marked by dots. Cystein residues are highlighted by bold C. Dashes mark gaps optimizing sequence alignment. The N-terminal signal sequence cleavage site is indicated by a solid inverted triangle. Slashes denote the reported cleavage sites of the C-terminal propeptides. The open box shows the position of the TGDC peptide domain. Arrows show the positions of Glu85, Asp98, Asp103, Asp186, Phe91, and Phe96. The amino acid sequence identity of the proteins compared with LIN1 is shown in the last column (%). The sources of the proteins and GenBank accession numbers are *LIN1*: *Linum usitatissimum*, AY181253; OSML13: *Solanum commersonii*, P50701; OLP: *Capsicum annuum*, CAC34055.2; P-23: *Lycopersicon esculentum*, Q01591; PR-5b: *Cicer arietinum*, CAA09228; osmotin: *Nicotiana tabacum*, S40046.1; CAOSM1: *Capsicum annuum*, AY262059; PR-5d: *Nicotiana tabacum*, M64081; thaumatin: *Thaumatococcus daniellii*, P02883.

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Linusitin  1 ARFDIQNKCPYTVWAASVPVGGGRQLNSGQTWXIDAP
LIN1 cDNA  1 AVIDIFNNCPYTVWAASTPIGGGRQLDHGQTWTIYPP
LIN1       1 AVIDI----------------------------
consensus  1 *..** . ......... ........  .... .  .
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Fig. 4 Comparison of the N-terminal sequence of linusitin from flax seeds, deduced LIN1 from cDNA, and linusitin-like protein (LIN1) from flax radicles. Identical amino acids are highlighted in black and marked by asterisks, and similar amino acids are highlighted in gray and marked by dots.

fluorescein-labeled *LIN1* cDNA (Gene Images Random-Prime Labeling System, Amersham Biosciences). Following hybridization, the blot was washed twice for 20 min at 65°C with 2X SSC, 0.1% SDS and twice for 15 min at 65°C with 0.5X SSC, 0.1% SDS. Hybridized fragments were detected using a Gene Images Detection System (Amersham Biosciences).

Northern Blot Analysis

Total RNA was extracted from liquid nitrogen–frozen tissues of flax with 4-d-old (seedling shoot and root) or 20-d-old (leaves, stem, and root) as described by Chomczynski and Sacchi (1987). Denatured aliquots of 20 μg RNA were fractionated on 1.5% formaldehyde agarose gel and transferred into HyBond-N+ membranes (Amersham Biosciences). Membranes were prehybridized for 1 h at 42°C in solution containing 50% formamide, 6X SSPE, 5X Denhardt's reagent, 0.5% SDS, and denatured salmon sperm DNA. The blots were hybridized in the same solution for 24 h at 42°C with a ^{32}P-labeled PCR fragment corresponding to the 180-bp fragment. A radioactive probe was prepared by random primer labeling with a Random Primed DNA Labeling Kit (Roche). Blots were washed three times in the following solutions: 2X SSPE (sodium chloride–sodium phosphate—EDTA solution), 0.1% SDS (5 min), 1X SSPE, 0.1% SDS at 50°C (10 min), and 0.1X SSPE, 0.1% SDS (5 min, 50°C). After washing, the membranes were exposed to x-ray films (X OMAT-AR; Kodak) with intensifying screens at −70°C for 24 h.

Results

Cloning and Sequence Analysis of a LIN1 *cDNA*

A cDNA library constructed from flax seedlings was screened using a radioactively labeled probe. A full-length cDNA clone was isolated. The clone was named *LIN1* cDNA, and its 964 nucleotides were sequenced (GenBank accession no. AY181253). The sequence of amplified 180-bp probe was identical to the part of the *LIN1* cDNA. The *LIN1* cDNA contains an open reading frame of 789 nucleotides, starting with an ATG codon at position 44 and ending with a TAG codon at position 833. The 3′ untranslated region is 131 nucleotides long. It includes the putative polyadenylation signal and poly A track (fig. 1).

The putative LIN1 protein contains an N-terminal signal peptide of 24 amino acid residues and a C-terminal extension signal peptide of 30 amino acid residues. The predicted molecular mass of the mature LIN1 is 23 kDa, and its pI value is 4.78.

Analysis of LIN1 at the Gene Level

Comparison of *LIN1* cDNA with the genomic fragment revealed 100% identity in nucleotide sequence (data not shown), which demonstrates that there is no intron interrupting the coding sequence. Southern hybridization analysis was prepared using genomic DNA digested by restriction enzymes that do not cleave within the coding sequence of *LIN1* and also using fluorescein-labeled *LIN1* cDNA as a probe. The results indicate that LIN1 is encoded by few gene copies (fig. 2).

Sequence Comparison with Related Proteins

The deduced amino acid sequence of LIN1 was aligned with sequences of seven osmotin-like proteins that show the strongest similarity to LIN1. The mature predicted proteins showed an identity of 72%–65% with osmotin-like proteins and 53% with thaumatin (fig. 3). LIN1 share the highest degree of identity (72%) with basic protein OSML13 from *Solanum commersonii*.

Sequence Comparison with Linusitin from Flax

The predicted mature N-terminus of LIN1, starting at Ala^{25}, differs in 11 of the 37 amino acids reported for the N-terminal sequence of linusitin from flax seeds (fig. 4). The occurrence of two different osmotin-like proteins in flax was additionally confirmed by comparing the first five amino acids of the N-terminal sequences of LIN1 (AVIDI) purified from the radicles (fig. 5) with linusitin (ARFDI) (fig. 4).

Expression Analysis of LIN1 Gene in Flax Tissues

RNA blotting was performed to analyze the organ specificity of *LIN1* expression in different flax tissues. The *LIN1* gene was found to be expressed constitutively in healthy

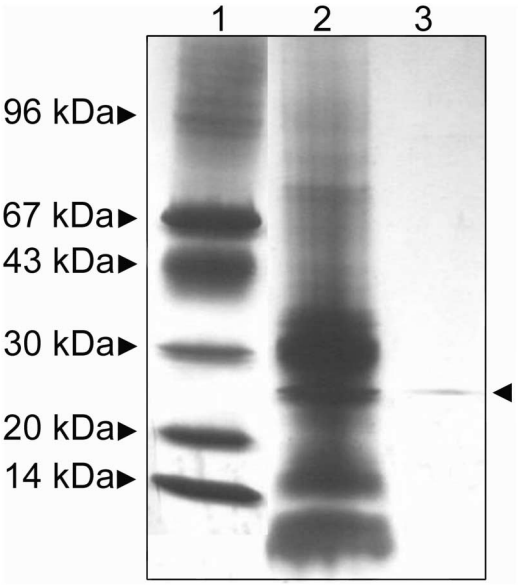

Fig. 5 SDS-PAGE patterns. Electrophoresis conditions are described in the text. 1 = protein molecular mass markers, 2 = crude extract, 3 = purified LIN1.

roots (fig. 6). A very low level of LIN1 mRNA was transcribed in upper parts of seedlings (fig. 6).

Discussion

We report the first isolation of an osmotin-like protein cDNA from flax, which contains an open reading frame of 789 nucleotides. The deduced amino acid sequence includes a signal peptide that comprises the first 24 amino acids (fig. 1). Although the pattern of this signal sequence is not entirely in agreement with the rules of von Heijne (1983), the position of Ala^{25}, instead of an amino acid with an ionic side chain (von Heijne 1983), is conserved among PR-5 proteins (figs. 1, 3). All 16 cysteine residues that are involved in disulfide bonds in PR-5 proteins (Anžlovar and Dermastia 2003) are conserved in LIN1 (fig. 3). Furthermore, the open reading frame of the *LIN1* cDNA encodes a putative C-terminal signal propeptide. It is composed of 30 amino acids and comprises an unusual stretch of seven consecutive asparagine residues (fig. 1). Although it is impossible to find a consensus for the C-terminal extension from different proteins (Vitale and Raikhel 1999), it has been suggested that the C-terminal signal propeptide mediates vacuolar targeting of PR-5 proteins (Melchers et al. 1993; Sato et al. 1995; Liu et al. 1996).

Comparison of the deduced LIN1 amino sequence with those of other PR-5 proteins indicated a close relationship of LIN1 with vacuolar basic osmotin-like proteins, but the pI value calculated from the deduced LIN1 amino acid sequence was acidic. Similarly, Hong et al. 2004 described the comparable sequence characteristics of basic osmotin-like proteins for an acidic PR-5 protein CAOSM1 from pepper. The differences between the calculated pIs and the characteristics of the isolated proteins might demonstrate the occurrence of some post-translational modification (e.g., deamidation) of the proteins. Nevertheless, it has also been suggested that the acidic osmotin-like proteins with the C-terminal extension signal sequence have the roles in plant cells that may be different from those of other acidic thaumatin-like proteins (Hong et al. 2004).

The flax LIN1 coding sequence is not interrupted by introns. Similar intronless sequences have also been reported for genes encoding PR-5 proteins from tobacco (Sato et al. 1996), wild potato (Zhu et al. 1995), *Benincasa hispida* (Shih et al. 2001), and black nightshade (Campos et al. 2002). One of the reasons why PR-5 protein genes have no introns could be to facilitate translation to proteins during the defence process.

Southern blot analysis of genomic DNA indicated that LIN1 was encoded by few copies in the flax genome. Variation in signal intensity between the hybridization bands indicated either the presence of multiple copies per restriction enzyme fragments or sequence diversity. *PR-5* multigene families have already been shown for several *Solanum* species and for oat (Campos et al. 2002). The reason why plants produce a large number of diverse PRs is not clear. One likely cause could be evolutionary pressure toward protection of the plant against pathogens and abiotic stress, since different *PR-5* genes are activated by different signals, e.g., ABA, ethylene, auxin, infection, abiotic stresses. In contrast, some other osmotin-like proteins are encoded by a single copy gene (Capelli et al. 1997; Shih et al. 2001).

A common trait shared by PR-5 proteins is their antifungal activity (Anžlovar and Dermastia 2003). The part of the molecules suggested to be responsible for the antifungal activity was revealed from the crystal structure of several PR-5 proteins (Batalia et al. 1996; Koiwa et al. 1999; Min et al. 2004). All PR-5 proteins with proved antifungal activity have an acidic cleft formed by Glu85, Asp98, Asp103, and Asp186 (Anžlovar and Dermastia 2003). These residues are also conserved in LIN1 (fig. 3). An additional feature of osmotin-like proteins, including LIN1, is a local hydrophobic patch at the edge of the cleft region, formed by two phenylalanine residues, Phe91 and Phe96 (fig. 3). The surface hydrophobicity was suggested to be involved in the interaction between fungal and plant cell membranes (Koiwa et al. 1999). Notably, these phenylalanine residues are replaced by tyrosines in thaumatin, which is not active against fungi (Ogata et al. 1992; Koiwa et al. 1999). The presence of the acidic cleft and phenylalanine residues in LIN1 may indicate that the protein has an antifungal activity.

Comparison of the deduced *LIN1* amino sequence with the reported N-terminal sequence of linusitin from flax seeds (Borgmeyer et al. 1992) revealed disagreement in 11 of the 37 amino acids. The sequence discrepancy was further confirmed by comparing the first five amino acids (AVIDI) of the N-terminal sequences of LIN1 purified from the radicles, with the corresponding sequence (ARFDI) in linusitin. Our results demonstrate unambiguously the presence of at least two osmotin-like proteins in flax tissues.

Several results have shown that constitutive osmotin-like protein gene expression is tissue specific (Anžlovar and Dermastia 2003). However, the *LIN1* mRNA was expressed predominantly in the roots of seedlings and mature plants (fig. 6). Additionally, a very weak *LIN1*-like signal was detected in the upper parts of seedlings 4 d after germination, but it disappeared after 20 d of growth (fig. 6). A similar prevailing root expression was reported for *CAOSM1* mRNA from *Capsicum annuum* (Hong et al. 2004), *PhOSM* mRNA from

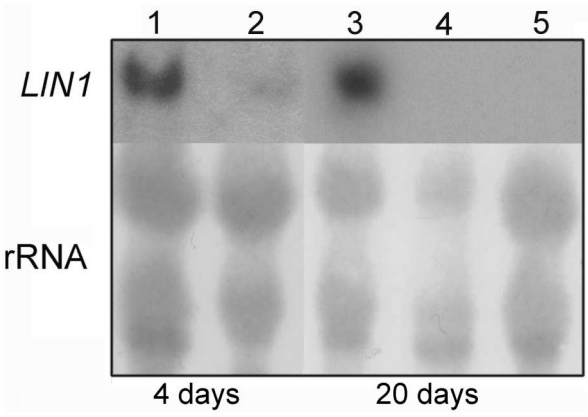

Fig. 6 Expression patterns of *LIN1* transcripts in various tissues of flax 4 d and 20 d after germination: 1 = seedling roots, 2 = seedling shoots, 3 = roots, 4 = stems, 5 = leaves. Equal loading of RNA was confirmed by staining the same blot with methylene blue.

Petunia hybrida (Kim et al. 2002), and PR-5d protein from tobacco (Koiwa et al. 1994). All these proteins share high sequence similarity with LIN1 (fig. 3) (Anžlovar and Dermastia 2003). On the contrary, in the proteins with less homology with LIN1, such as PR-5 protein BFTP from *Brassica campestris* (Cheong et al. 1997) or the osmotin-like protein from *Arabidopsis thaliana* (Capelli et al. 1997), very low levels of their mRNA were present in roots but were expressed mostly in the upper parts of plants, including flowers.

To conclude, LIN1 is an osmotin-like protein, being a member of a small family of *PR-5* genes in flax, including linusitin (Anžlovar et al. 1998). The presence of LIN1 in radicles of the germinated flax plants and in mature roots, together with the occurrence of linusitin in flax seeds, suggests that both proteins have an important role in defence during seed development, germination, and later in the rhizosphere.

Acknowledgments

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Literature Cited

Altschul SF, TL Madden, AA Schäffer, J Zhang, Z Zhang, W Miller, DJ Lipman 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402.

Anžlovar S, M Dalla Serra, M Dermastia, G Menestrina 1998 Membrane permeabilizing activity of pathogenesis-related protein linusitin from flax seed. Mol Plant-Microbe Interact 7:610–617.

Anžlovar S, M Dermastia 2003 The comparative analysis of osmotins and osmotin-like PR-5 proteins. Plant Biol 5:116–124.

Batalia MA, AF Monzingo, S Ernst, W Roberts, JD Robertus 1996 The crystal structure of the antifungal protein zeamatin, a member of the thaumatin-like, PR-5 protein family. Nat Struct Biol 3:19–23.

Bjellqvist B, GJ Hughes, C Pasquali, N Paquet, F Ravier, J-C Sanchez, S Frutiger, DF Hochstrasser 1993 The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. Electrophoresis 14:1023–1031.

Borgmeyer JR, CE Smith, QK Huynh 1992 Isolation and characterization of a 25 kDa antifungal protein from flax seeds. Biochem Biophys Res Commun 187:480–487.

Campos MA, SG Ribeiro, DJ Rigden, DC Monte, MF Grossi de Sá 2002 Putative pathogenesis-related genes within *Solanum nigrum* L. var. *americanum* genome: isolation of two genes coding for PR5-like proteins, phylogenetic and sequence analysis. Physiol Mol Plant Pathol 61:205–216.

Capelli N, T Diogon, H Greppin, P Simon 1997 Isolation and characterization of a cDNA clone encoding an osmotin-like protein from *Arabidopsis thaliana*. Gene 191:51–56.

Cheong NE, YO Choi, WY Kim, SC Kim, MJ Cho, SY Lee 1997 Purification of an antifungal PR-5 protein from flower buds of *Brassica campestris* and cloning of its gene. Physiol Plant 101: 583–590.

Chomczynski P, N Sacchi 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. Anal Biochem 162:156–159.

Cvetkovic A, S Gorjanovic, J Hranisavljevic, D Vucelic 1997 Isolation and characterization of pathogenesis-related proteins from brewer's barley grain. J Serb Chem Soc 62:51–56.

Fils-Lycaon R, PA Wiersma, KC Eastwell, P Sautiere 1996 A cherry protein and its gene, abundantly expressed in ripening fruit, have been identified as thaumatin-like. Plant Physiol 111:296–273.

Hong JK, HW Jung, BK Lee, SC Lee, YK Lee, BK Hwang 2004 An osmotin-like protein gene, *CAOSM1*, from pepper: differential expression and in situ localization of its mRNA during pathogen infection and abiotic stress. Physiol Mol Plant Pathol 64:301–310.

Jeanmougin F, JD Thompson, M Gouy, DG Higgins, TJ Gibson 1998 Multiple sequence alignment with Clustal X. Trends Biochem Sci 23:403–405.

Kim H, J-H Mun, BH Byun, H-J Hwang, YM Kwon, S-G Kim 2002 Molecular cloning and characterization of the gene encoding osmotin protein in *Petunia hybrida*. Plant Sci 162:745–752.

Koiwa H, H Kato, T Nakatsu, J Oda, Y Yamada, F Sato 1999 Crystal structure of tobacco PR-5d protein at 1.8 Å resolution reveals a conserved acidic cleft structure in antifungal thaumatin-like proteins. J Mol Biol 286:1137–1145.

Koiwa H, F Sato, Y Yamada 1994 Characterization of accumulation of PR-5 proteins by IEF-immunoblot analysis. Plant Cell Physiol 35: 821–827.

Liu D, D Rhodes, MP D'Urzo, Y Xu, ML Narasimhan, PM Hasegawa, RA Bressan, L Abad 1996 In vivo and in vitro activity of truncated osmotin that is secreted into the extracellular matrix. Plant Sci 121:123–131.

Melchers LS, MB Sela-Buurlage, SA Vloemans, CP Woloshuk, JSCV Roekel, PJM van den Elzen, BJC Cornelissen 1993 Extracellular targeting of the vacuolar tobacco proteins AP24 chitinase and β-1,3-glucanase in transgenic plants. Plant Mol Biol 21:583–593.

Min K, SC Ha, PM Hasegawa, RA Bressan, D Yun, KK Kim 2004 Crystal structure of osmotin, a plant antifungal protein. Proteins 54: 170–173.

Nielsen H, J Engelbrecht, S Brunak, G von Heijne 1997 Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng 10:1–6.

Ogata M, PF Gordon, AM de Vos, SH Kim 1992 Crystal structure of a sweet tasting protein, thaumatin I, at 1.65 Å resolution. J Mol Biol 228:893–908.

Rogers SW, AJ Bendich 1985 Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol Biol 5:69–76.

Salzman RA, I Tikhonova, BP Bordelon, PM Hasegawa, RA Bressan 1998 Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense response during ripening in grape. Plant Physiol 117:465–472.

Sambrook J, EF Fritsch, T Maniatis 1989 Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sato F, S Kitajima, T Koyama, Y Yamada 1996 Ethylene-induced gene expression of osmotin-like protein, a neutral isoform of tobacco PR-5, is mediated by the AGCCGCC cis-sequence. Plant Cell Physiol 37:249–255.

Sato F, H Koiwa, Y Sakai, N Kato, Y Yamada 1995 Synthesis and secretion of tobacco neutral PR-5 protein by transgenic tobacco and yeast. Biochem Biophys Res Commun 211:909–913.

Shih C-Y, J Wu, S Jia, AA Khan, K-L-H Ting, DS Shih 2001 Purification of an osmotin-like protein from the seeds of *Benincasa hispida* and cloning of the gene encoding this protein. Plant Sci 160: 817–826.

Van der Wel H, K Loeve 1972 Isolation and characterization of thaumatin 1 and 2, the sweet-tasting proteins from *Thaumatococcus daniellii* Benth. Eur J Biochem 31:221–225.

Van Loon LC, EA Van Strien 1999 The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol 55:85–97.

Vitale A, NV Raikhel 1999 What do proteins need to reach different vacuoles? Trends Plant Sci 4:149–155.

von Heijne G 1983 Patterns of amino acids near signal-sequence cleavage sites. Eur J Biochem 133:17–21.

Zhu B, THH Chen, PH Li 1995 Activation of two osmotin-like protein genes by abiotic stimuli and fungal pathogen in transgenic potato plants. Plant Physiol 108:929–937.