PHYSIOLOGIA PLANTARUM 86: 245–252. Copenhagen 1992

Lectins of members of the Amaryllidaceae are encoded by multigene families which show extensive homology

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Van Damme, E. J. M., Goldstein, I. J., Vercammen, G., Vuylsteke, J. and Peumans, W. J. 1992. Lectins of members of the Amaryllidaceae are encoded by multigene families which show extensive homology. – Physiol. Plant. 86: 245–252.

Screening of complementary DNA (cDNA) libraries constructed from poly(A) rich RNA isolated from young developing ovaries of *Narcissus* cv. Fortune and *Hippeastrum* hybr. resulted in the isolation of multiple lectin cDNA clones. Although the lectin clones show a high degree of overall homology within their coding region, they clearly differ from each other at some positions in their nucleotide sequence and deduced amino acid sequence. Moreover, since some differences in the sequence result in different charges along the lectin polypeptides, the different cDNA clones encode lectins with different isoelectric points, which explains the occurrence of multiple lectin isoforms in *Narcissus* and *Hippeastrum* at the molecular level. Furthermore, Southern blot analysis of genomic DNA isolated from *Narcissus* and *Hippeastrum* yielded numerous restriction fragments hybridizing with lectin cDNA probes leaving no doubt that the *Narcissus* and *Hippeastrum* lectins, like the *Galanthus nivalis* lectin, are encoded by families of closely related lectin genes.

Key words – Amaryllidaceae, cDNA cloning, isoform, lectin, multigene family.

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Introduction

In 1987 we reported the isolation and characterization of an apparently new type of lectin from the bulbs of snowdrop (*Galanthus nivalis* L.) with exclusive specificity towards mannose (Van Damme et al. 1987). Since then it has been shown that all representatives of the family Amaryllidaceae (Van Damme et al. 1988) contain high concentrations of similar mannose-specific lectins, at least during certain developmental stages (Van Damme and Peumans 1990b). Although all these Amaryllidaceae lectins exhibit an exclusive specificity towards mannose and resemble each other with respect to their biochemical and serological properties, several marked differences have been observed between the agglutinins from snowdrop (*Galanthus nivalis*), daffodil (*Narcissus pseudonarcissus*) and amaryllis (*Hippeastrum* hybr.). First, there are obvious differences in molecular structure as the snowdrop and the amaryllis lectins are tetramers whereas the daffodil lectin is a dimer. Second, the fine specificity of the 3 Amaryllidaceae lectins studied thus far is different. The snowdrop lectin is highly specific for terminal α-1,3-linked mannose oligomers (Shibuya et al. 1988a) whereas the daffodil lectin has the highest affinity for both terminal and internal α-1,6-linked mannosyl residues, and the amaryllis agglutinin binds both α-1,3- and α-1,6-linked mannosyl residues (Kaku et al. 1990). Third, the antiretroviral activities of the lectins from snowdrop, daffodil and amaryllis definitely differ in the sense that the relative inhibitory potency of the 3 agglutinins varies as a function of the virus and the assay system (Balzarini et al. 1991). It is evident, therefore, that there are differences in the structure of the carbohydrate binding site of the 3 lectins which ultimately result from differences in the amino acid sequences of the lectin polypeptides.

Irrespective of the obvious differences between the individual members of the Amaryllidaceae lectins an

Received 27 March, 1992; revised 26 June, 1992

increasing interest is drawn to this group of agglutinins for several reasons. First, because of their unique carbohydrate binding specificity Amaryllidaceae lectins are successfully exploited for the purification and characterization of glycoconjugates (Shibuya et al. 1988b). Second, since they are potent inhibitors of human and animal retroviruses including human immunodeficiency virus, the Amaryllidaceae lectins have become important tools in the research on AIDS (Balzarini et al. 1991). Third, the recent discovery that the snowdrop lectin exhibits a pronounced toxicity towards several species of both chewing and sucking insects (Hilder et al. 1991) has raised the hope of a possible use of Amaryllidaceae lectins for plant protection purposes. Since, unlike most of plant agglutinins the Amaryllidaceae lectins have no toxic effects on rats upon oral administration, they certainly are also of potential interest from this point of view (Pusztai et al. 1990).

Recently we reported the isolation and characterization of the snowdrop lectin genes (Van Damme et al. 1991b). Since the present interest in the whole group of Amaryllidaceae lectins on the one hand and the obvious differences between the individual members of this class of proteins on the other hand justify a detailed comparative analysis at the molecular level, we isolated and characterized the daffodil and amaryllis lectin genes. In this paper we present evidence that like the *Galanthus nivalis* agglutinin, the lectins from *Narcissus* cv. Fortune and *Hippeastrum* hybr. also are encoded by multiple genes. Moreover it is shown that all cDNA clones show extensive homology indicating that there is not only a high degree of sequence homology between the different isoforms of one species but also between (iso)lectins of different Amaryllidaceae species.

Abbreviations – kb, kilobase; SSC, solution containing 0.9 *M* sodium chloride and 0.09 *M* sodium citrate, pH 7.0.

The nucleotide sequences reported in this paper have been deposited with the Genbank TM/EMBL Data library and are available under accesssion numbers M88117, M88118, M88119, M88120, M88121, M88122, M88123, M88124, M88125, M88126, M88127, M88128, M88129, M88130, M88131, M88132 and M88133.

Materials and methods

Plant material

Young developing ovaries of *Narcissus* cv. Fortune and *Hippeastrum* hybr. were collected at the onset of flowering and frozen at −80°C before extraction of nucleic acids.

RNA isolation and construction of complementary DNA (cDNA) libraries

Poly(A) RNA was isolated from the ovaries of *Narcissus* cv. Fortune and *Hippeastrum* hybr. via the hot phenol method followed by affinity chromatography on oligo-dT cellulose as reported previously (Van Damme et al. 1991c). This poly(A) RNA was then used in the cDNA synthesis kit from Kabi-Pharmacia (Uppsala, Sweden) to construct the cDNA libraries. The cDNA was inserted into the Eco RI site of the multifunctional phagemid pT7T3 18U (Kabi-Pharmacia, Uppsala, Sweden) and propagated in *Escherichia coli* XL1 Blue cells (Stratagene, La Jolla, CA, USA).

Screening of cDNA libraries

Recombinant lectin clones were screened by colony hybridization using the ^{32}P-labeled cDNA insert LECGNA 6 (See Fig. 1) from snowdrop as a probe. Hybridization was carried out for 24 h at 68°C in 2 × SSC (1 × SSC = 0.9 *M* sodium chloride and 0.09 *M* sodium citrate, pH 7.0), 10 × Denhardt's solution (Denhardt et al. 1978), 0.1% (w/v) SDS, 100 μg ml^{-1} denatured salmon sperm DNA and 10 μg ml^{-1} poly(A). After prehybridization for 4 h the labeled cDNA insert (40 kBq ml^{-1}) was added to this mixture. Following hybridization filters were washed consecutively in 2 × SSC containing 0.1% (w/v) SDS (4 times for 10 min at 68°C) and 1 × SSC containing 0.1% (w/v) SDS (twice for 10 min at 68°C), and exposed to FUJI film at −80°C. Plasmids were isolated from purified single colonies using the alkaline lysis method described by Mierendorf and Pfeffer (1987) and sequenced by the dideoxy method (Sanger et al. 1977).

Northern blot analysis

Approximately 3 μg of poly(A) RNA was denatured in glyoxal and DMSO and separated in a 1.2% (w/v) agarose gel, according to Maniatis et al. (1982). Following electrophoresis the RNA was transferred to Immobilon N membranes (Millipore, Bedford, USA) and hybridized under the same conditions as reported previously (Van Damme et al. 1991b) using a ^{32}P-labeled cDNA insert as a probe. An RNA ladder (0.16–1.77 kb) was used as a marker.

Southern blot analysis

Total DNA from *Narcissus* cv. Fortune and *Hippeastrum* hybr. ovaries was isolated according to the procedures described by Dellaporta et al. (1983) and de Kochko (1990). Approximately 30 μg of DNA was digested with restriction endonucleases and electrophoresed in a 0.8% (w/v) agarose gel. DNA was transferred to Immobilon N membranes and hybridized using a ^{32}P-labeled cDNA insert from daffodil (LECNPA 3, Fig. 1) or amaryllis (LECHHA 2, Figs 1 and 2) as a probe (Van Damme et al. 1991c). Hybridization was carried out at 68°C for 16 h in 6 × SSC containing 0.5% (w/v) SDS, 5 × Denhardt's solution (Denhardt et al. 1978) and 100 μg ml^{-1} denatured salmon sperm DNA.

After prehybridization for 4 h the labeled DNA (40 kBq ml^{-1}) was added to this mixture. Following hybridization the membrane was washed consecutively in 2 × SSC (30 min at 68°C), 2 × SSC containing 0.1% (w/v) SDS (30 min at 68°C) and 0.5 × SSC (10 min at 68°C).

Computer analyses

Desoxyribonucleic acid and protein sequences were analyzed using programs from PCGene (Biomed, Geneva, Switzerland) and Genepro (Riverside Scientific, Seattle, USA).

Results

Isolation and characterization of lectin cDNA clones from *Narcissus* cv. Fortune and *Hippeastrum* hybr.

cDNA libraries were constructed from RNA isolated from young developing ovaries of *Narcissus* cv. Fortune and *Hippeastrum* hybr. which are known to contain high lectin concentrations at this stage of development (Van Damme and Peumans 1990b). Initially these cDNA libraries were screened under very stringent conditions (68°C, 5.4 *M* salt) using a nick translated ^{32}P-labeled cDNA clone LECGNA 6 isolated from *Galanthus nivalis* (Van Damme et al. 1991c). In a later stage cDNA clones from either daffodil or amaryllis were used to probe the respective libraries. About 6 and 0.5% of the bacterial colonies transformed with the cDNA from *Narcissus* and *Hippeastrum*, respectively, reacted positively with LECGNA 6 indicating that they contained lectin sequences. This high number of positive colonies is not surprising since the lectin is an abundant protein in young ovaries. The fact that the amaryllis cDNA library contains less lectin cDNA clones compared to the library constructed for daffodils is in good agreement with the results from northern blot analysis (Fig. 2) which show a much weaker signal for amaryllis after probing with a lectin cDNA insert, indicating that less lectin mRNA was present in the ovary at the time of sampling of the plant material.

Multiple cDNA clones were isolated from each cDNA library which clearly differ from each other at certain positions within both their nucleotide and deduced amino acid sequence (Fig. 1). The lectin cDNA sequences all contain an open reading frame encoding a preprolectin which besides the coding sequence of the mature lectin also encodes both a signal peptide and a C-terminal peptide which are removed co- and post-translationally, respectively. The cleavage site of the signal peptide, determined both by application of the rules of von Heijne (1986) and by comparison of the deduced amino acid sequence of the lectin clones to the N-terminal lectin sequence (I. J. Goldstein, unpublished data), is located between amino acid 24 and 25. This position coincides with the most probable cleavage site for the signal peptide in snowdrop lectin clones (Van Damme et al. 1991c). Because of the high sequence homology between the different isolectin clones of all Amaryllidaceae species and similar results from in vivo and in vitro biosynthesis studies of the lectin we assume that the C-terminal peptide of the daffodil and amaryllis clones will also be cleaved at the same position (after the amino acid sequence Thr-Gly of the preproprotein) as in snowdrop lectin.

The amino acid sequences of the lectin cDNA clones isolated from daffodil (LECNPA 1–7) and amaryllis (LECHHA 1–8) are shown in Fig. 1 and compared to the sequence of two snowdrop lectin clones LECGNA 1 and 2 representing the two classes of cDNA sequences found in snowdrop as reported previously (Van Damme et al. 1991c). All sequences are aligned to obtain maximum homology and compared to the sequence of LECNPA 1.

The signal peptides of all lectin clones isolated show a high degree of homology. All daffodil lectin clones except LECNPA 7 contain an additional amino acid at position 19 compared to the lectin clones of amaryllis and snowdrop, resulting in a signal peptide of 24 amino acids in stead of 23 amino acids. None of the lectin clones isolated for the amaryllis lectin contained an ATG start codon. However, taking into account the high degree of homology between the signal sequences of all Amaryllidaceae lectin clones we assume that the start codon of the amaryllis lectin clones will be at approximately the same position as in the other lectin clones. Primer extension analyses on poly(A) RNA from daffodil and amaryllis revealed that the major transcription initiation site was located approximately 40 bases upstream from the initiation codon. However, several other but fainter signals up to 55 bases upstream from the AUG are present, which indicates some heterogeneity in the mRNA at the 5′ end (data not shown).

Whereas the sequences encoding the mature lectin polypeptides show a high degree of overall homology the lectin clones definitely differ from each other at certain positions within the sequences. It is obvious from Fig. 1 that some regions within the isolectin sequences are more conserved than others. Lectin clone LECHHA 4 apparently contains an additional amino acid within the sequence of the mature lectin polypeptide compared to the other amaryllis, the daffodil and the snowdrop lectin clones. Since some differences in amino acids create different charges along the lectin polypeptide these differences result in lectin polypeptides with differing isoelectric points ranging from 3.66 to 4.24 and from 3.90 to 5.45 for the mature lectin polypeptides of the daffodil and the amaryllis lectin, respectively, which is in good agreement with the results from isoelectric focusing experiments (Van Damme et al. 1991a). The calculated molecular weight of the mature lectin polypeptide varies between 11.6 and 11.9 kDa for both daffodil and amaryllis which is consistent with the molecular mass of 12.5 kDa for the lectin

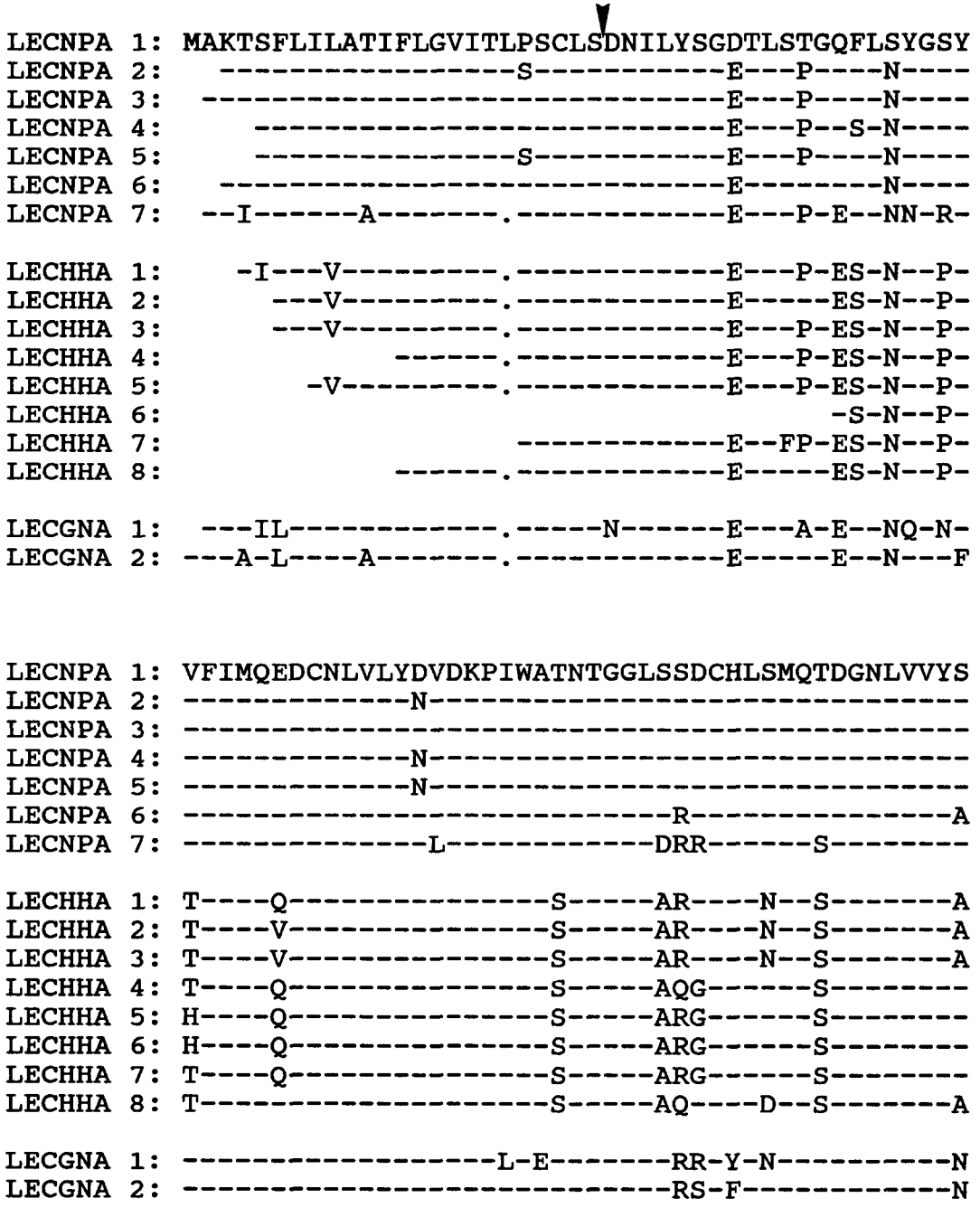
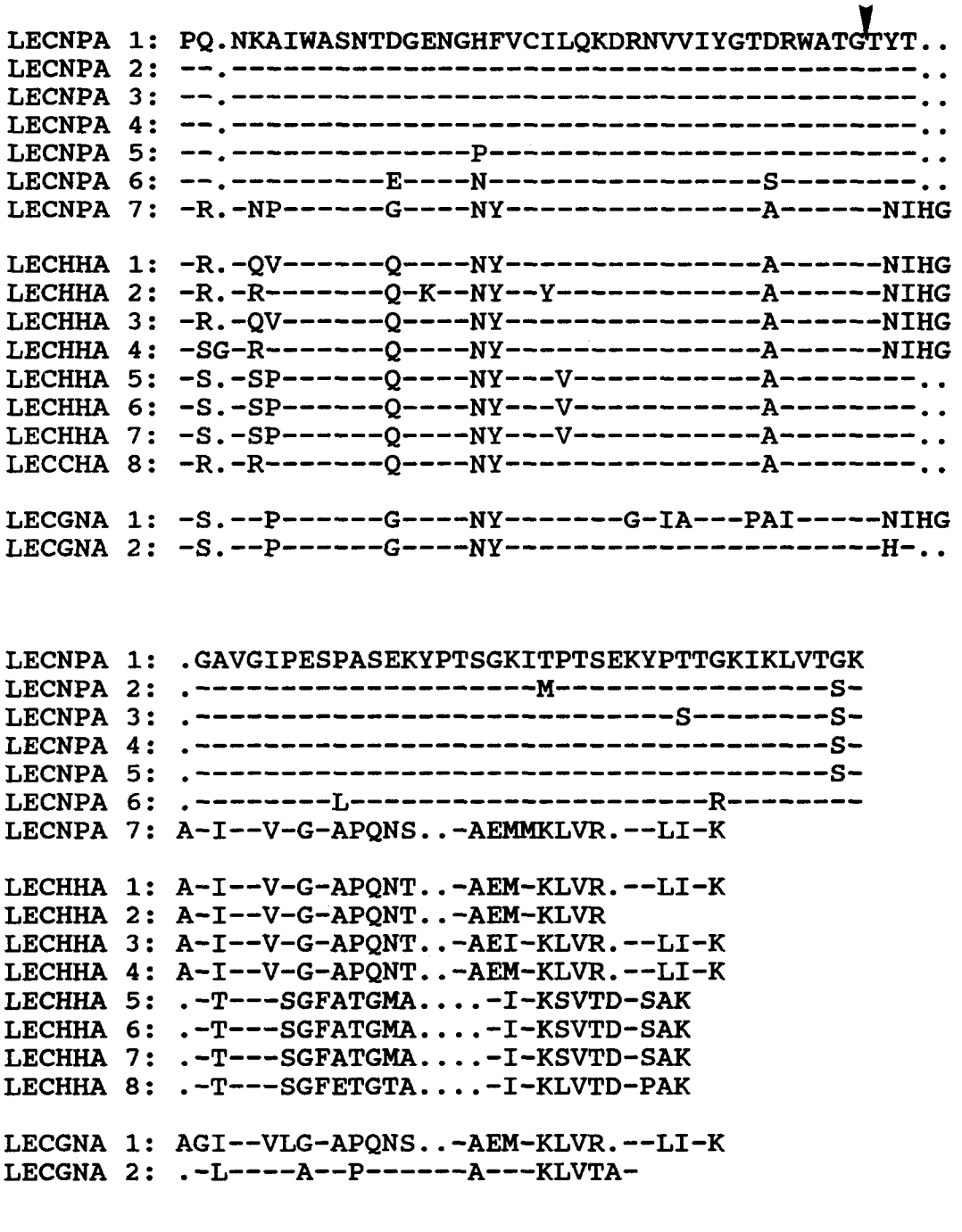


Fig. 1. Deduced amino acid sequence of different cDNA clones from daffodil, amaryllis and snowdrop. Arrowheads indicate the possible processing site for the cleavage of the signal peptide and the C-terminal peptide, respectively. (−) Denotes sequence identity with LECNPA 1. Dots represent gaps introduced for maximal alignment.


LECNPA 1: PQ.NKAIWASNTDGENGHFVCILQKDRNVVIYGTDRWATGTYT..
LECNPA 2: --.----------..
LECNPA 3: --.----------..
LECNPA 4: --.----------..
LECNPA 5: --.----------P----------..
LECNPA 6: --.---------E----N----------S--------..
LECNPA 7: -R.-NP------G----NY----------A------NIHG
LECHHA 1: -R.-QV------Q----NY----------A------NIHG
LECHHA 2: -R.-R-------Q-K--NY--Y----------A------NIHG
LECHHA 3: -R.-QV------Q----NY----------A------NIHG
LECHHA 4: -SG-R-------Q----NY----------A------NIHG
LECHHA 5: -S.-SP------Q----NY---V----------A--------..
LECHHA 6: -S.-SP------Q----NY---V----------A--------..
LECHHA 7: -S.-SP------Q----NY---V----------A--------..
LECCHA 8: -R.-R-------Q----NY----------A--------..
LECGNA 1: -S.--P------G----NY-------G-IA---PAI-----NIHG
LECGNA 2: -S.--P------G----NY----------H-..
LECNPA 1: .GAVGIPESPASEKYPTSGKITPTSEKYPTTGKIKLVTGK
LECNPA 2: .----------M----------S-
LECNPA 3: .----------S--------S-
LECNPA 4: .----------S-
LECNPA 5: .----------S-
LECNPA 6: .--------L----------R--------
LECNPA 7: A-I--V-G-APQNS..-AEMMKLVR.--LI-K
LECHHA 1: A-I--V-G-APQNT..-AEM-KLVR.--LI-K
LECHHA 2: A-I--V-G-APQNT..-AEM-KLVR
LECHHA 3: A-I--V-G-APQNT..-AEI-KLVR.--LI-K
LECHHA 4: A-I--V-G-APQNT..-AEM-KLVR.--LI-K
LECHHA 5: .-T---SGFATGMA....-I-KSVTD-SAK
LECHHA 6: .-T---SGFATGMA....-I-KSVTD-SAK
LECHHA 7: .-T---SGFATGMA....-I-KSVTD-SAK
LECHHA 8: .-T---SGFETGTA....-I-KLVTD-PAK
LECGNA 1: AGI--VLG-APQNS..-AEM-KLVR.--LI-K
LECGNA 2: .-L----A--P------A---KLVTA-

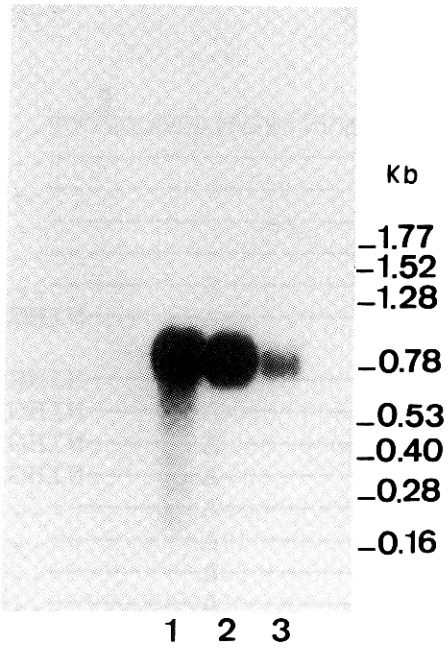


Fig. 2. Northern blot of poly(A) RNA isolated from ovaries of snowdrop (lane 1), daffodil (lane 2) and amaryllis (lane 3). The blot was hybridized using the ^{32}P-labeled cDNA insert from LECNPA 3.

polypeptides as determined by SDS-PAGE (Van Damme et al. 1988).

Compared to the signal peptide and the mature lectin polypeptide the C-terminal peptide of the isolectin clones shows a higher variability with respect to both its length and its sequence. However, all lectin sequences can be classified into a few groups depending on their C-terminal sequence. For instance, whereas the C-terminal peptide of LECNPA 7 certainly differs from all other daffodil lectin clones isolated, it is highly homologous to the C-terminal of LECHHA 1–4 and LECGNA 1. Only lectin clones belonging to this group contain a putative glycosylation site within their C-terminal peptide.

In contrast to the coding region of the different lectin clones the 3′ untranslated region of the different clones shows a high degree of heterogeneity. Although multiple putative polyadenylation sites could be identified there are only a few clones which contain a poly(A) tail.

Sequence homology between different Amaryllidaceae lectin clones

Sequence comparison for the total coding region of the lectin revealed that all cDNA clones encoding the daffodil lectin show between 70 and 98%, and between 78 and 99% homology in the coding region at the deduced amino acid and at the nucleotide level, respectively. Similarly all amaryllis lectin clones share between 70

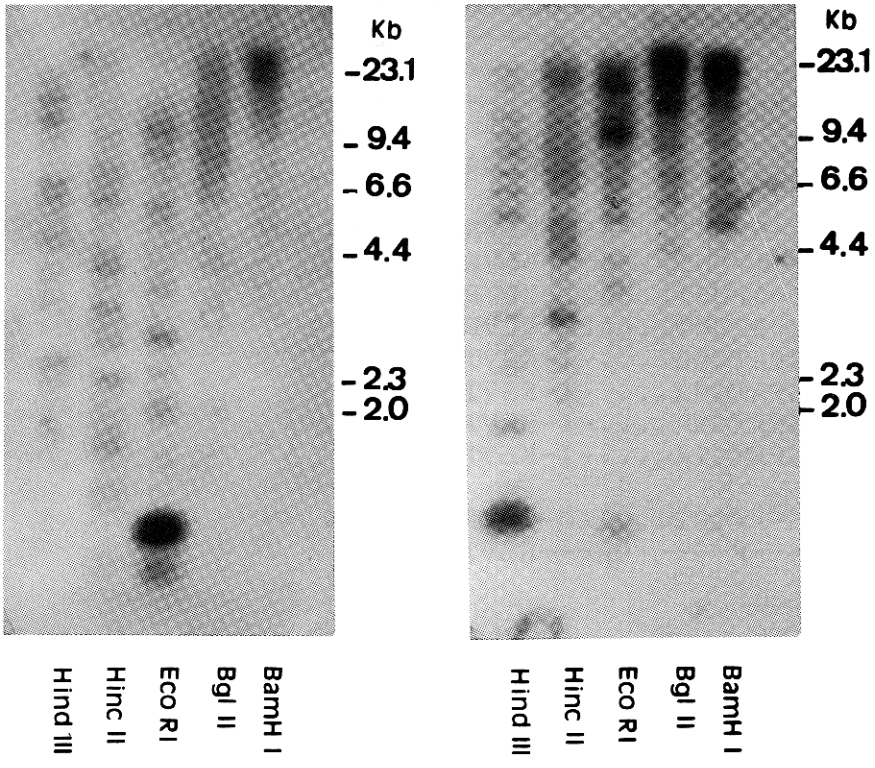


Fig. 3. Southern blot of genomic DNA isolated from daffodil (left) and amaryllis (right) ovaries. DNA was digested with Hind III, Hinc II, Eco RI, Bgl II and BamH I and hybridized with the labeled cDNA insert from LECNPA 3.

and 99%, and between 78 and 98% homology in their coding region at the deduced amino acid and the nucleotide level, respectively, whereas snowdrop lectin clones show between 69 and 94%, and between 82 and 96% homology in the coding region at the deduced amino acid level and the nucleotide level, respectively (Van Damme et al. 1991c). A comparison of the isolectin clones from different species reveals that daffodil and amaryllis lectin clones share between 66 and 88%, and between 70 and 86% similarity in their coding region at the deduced amino acid and at the nucleotide level, respectively. Compared to the snowdrop lectin clones, daffodil lectin clones show between 63 and 83%, and between 74 and 87% homology at the deduced amino acid level and at the nucleotide level whereas amaryllis lectin clones share between 60 and 80%, and between 68 and 82% similarity at the deduced amino acid and at the nucleotide level, respectively.

Northern blot analysis

Northern blot analysis was performed to assay the total length and size distribution of the lectin mRNA from daffodil and amaryllis and to compare them to snowdrop mRNA. As shown in Fig. 2 hybridization of the blot using a lectin cDNA insert from daffodil (LECNPA 3) revealed one band of lectin mRNA in each lane indicating that snowdrop, daffodil and amaryllis contain lectin mRNAs of the same size. This result was identical when hybridization was performed using a lectin cDNA insert from either snowdrop or amaryllis. On the basis of the electrophoresis pattern of the RNA markers the length of the lectin mRNA was estimated to be approximately 800 nucleotides which is consistent with the size of the cDNA clones.

Southern blot analysis

Digestion of genomic DNA isolated from *Narcissus* and *Hippeastrum* ovaries using various restriction enzymes (BamH I, Bgl II, Eco RI, Hinc II and Hind III) revealed a complex pattern of hybridizing bands when the blot was probed with labeled lectin cDNA insert LECNPA 3 and LECHHA 2, respectively (Fig. 3). Since Eco RI is the only restriction enzyme used that cleaves within the lectin insert these results suggest that there are multiple lectin genes which constitute a gene family.

Discussion

In a previous paper (Van Damme et al. 1991c) we reported the isolation and characterization of multiple cDNA clones for the *Galanthus nivalis* lectin. It was shown that although all lectin clones showed a high degree of homology they definitely differed from each other at certain positions within their nucleotide and deduced amino acid sequence. It can be expected that these differences are very important in view of the different carbohydrate binding specificities and biological activities of the lectins. Since these differences result in lectin polypeptides with different isoelectric points the occurrence of multiple lectin isoforms can be explained at the molecular level. In this paper we present evidence that the lectins from *Narcissus* cv. Fortune and *Hippeastrum*, two other representatives of the family Amaryllidaceae, also are encoded by multiple genes. Evidently, these results are consistent with the earlier observation that the isolectin pattern in different species and cultivars of *Narcissus* is genetically determined as was shown previously (Van Damme and Peumans 1990a).

A high degree of homology was found between the coding regions of cDNA clones encoding isolectins of different Amaryllidaceae species. However, the Amaryllidaceae lectins show a higher sequence variability within the 3′ untranslated region of their cDNA clones. This is in contrast with the cDNA clones encoding the wheat germ agglutinin isolectins (Smith and Raikhel 1989) and the patatin proteins (Mignery et al. 1984) which show a high degree of homology in both the coding region and the 3′ untranslated part of the clones, but is similar to the sequences reported for sporamin (Murakami et al. 1986) and glutamine synthetase (Sakamoto et al. 1989).

The concept of lectins being encoded by (extended) multigene families is quite new in lectin research though it is not unusual for a plant protein. Several multigene families have been reported within the plant kingdom e.g. for enzymes (α-amylase, Huttly et al. 1988, Sutliff et al. 1991) and for storage proteins in legumes (Lycett et al. 1983, Domoney and Casey 1990), maize (Hagen and Rubenstein 1981, Burr and Burr 1982, Pedersen et al. 1982), potato (Mignery et al. 1988) and cereals (Miflin et al. 1984). Earlier findings that all Amaryllidaceae lectins are present in high concentrations during certain stages of plant development and, in addition, are developmentally regulated like typical storage proteins (Van Damme and Peumans 1990b) suggested that these lectins might function as storage proteins. In view of the presumed storage role of the lectins in the plant it seems logical that they are encoded by a family of closely related genes. It should be emphasized, however, that Amaryllidaceae lectins can fulfil a second function besides their storage role. Since the lectin is only present at certain stages of development and occurs in almost all plant tissues it might also play an active role in plant defense. The recent discovery that at least the *Galanthus nivalis* agglutinin exhibits a pronounced toxicity against insects certainly points in this direction. Through the occurrence of multiple isoforms the protein could broaden its spectrum of activity.

Finally, the results reported here emphasize the evolutionary conservation of the Amaryllidaceae lectin genes. We find it striking that both at the amino acid and at the nucleotide levels the homology between the

lectin genes from *Galanthus nivalis* and *Narcissus pseudonarcissus* (which have their centre of origin in Europe and Western Asia) is not higher than that between the lectin genes of *Hippeastrum* hybr. and *Galanthus nivalis* or *Hippeastrum* hybr. and *Narcissus pseudonarcissus*, and this in spite of the fact that *Hippeastrum* hybr. has its centre of origin in America.

Acknowledgements – This work was supported in part by grant No 20059.89N from the National Fund for Scientific Research (Belgium). W.P. is Research Director and E.J.M.V.D. Research Assistant of this fund. A grant from the NIH (GM 29470) to I.J.G. is also acknowledged.

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Edited by C. H. Bornman