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Biodiversity of Mannose-Specific Lectins within *Narcissus* Species

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Mannose-specific lectins (MSLs) were isolated from the bulbs of 27 species of wild Spanish *Narcissi* and compared to the commercially available MSL from daffodil (*Narcissus pseudonarcissus,* NPA). Molecular weight analysis showed the monomers of all the MSLs were at, or around, 12.5 kD. Haemagglutination assays showed that the MSLs exhibited activities at up to four times greater than that displayed by NPA and other MSLs derived from other species such as *Galanthus nivalus* (snowdrop) and *Allium ursinum* (ramson). Elution profiles from ion exchange chromatography exhibited similarities for species within the same taxonomic section suggesting that this method could aid in species classification. Further analysis by isoelectric focusing showed many isolectins are present in vivo and that even within a single peak from ion exchange chromatography there are numerous isolectins present. The basis of the isolectin heterogeneity is suggested to reside in the tetraploidy (sometime triploidy) nature of *Narcissus* genes.

KEYWORDS: Mannose-specific; lectin; narcissus; HIV; isolectin; agglutination; mannosylation

INTRODUCTION

Growing awareness of the ubiquity and structural diversity of glycoproteins conserved throughout protein evolution (*1, 2*), together with the realization that oligosaccharide structures of glycoproteins sometimes undergo dramatic changes during differentiation and in pathological processes, has intensified the search for the biological roles of glycosylation (*3, 4*).

The reported biological roles of the poly/oligosaccharides on glycoproteins are varied, with the following four main functions attributed to them: (a) aiding in the conformation and stability of proteins (*5*); (b) controlling the half-lives of proteins and cells (*6, 7*); (c) providing ligands for specific binding events mediating protein targeting, cell matrix, and/or cell−cell interactions (*8*) including intracellular sorting and quality control (*2*); and (d) functioning as receptor molecules and/or target structures of microorganisms such as yeast and bacteria (*9*−*12*), toxins (*13, 14*), viruses (*15, 16*) and antibodies (*17*), and also facilitating the masking of target structures (*18*).

The importance of the nature and extent of glycosylation has meant that these regions are now the subject of many diverse and intense research efforts. For example, the functional and structural analyses of the glycosylated regions may provide an avenue to novel disease therapies. However, such an undertaking requires that the polysaccharide region is fully characterized. An emergent biochemical tool in this area is the use of lectins, mainly derived from plants. Lectins are "proteins which contain at least one non-catalytic domain, which binds reversibly to a specific mono- or oligosaccharide" (*19*).

Plant-derived lectins exhibit a diverse range of specificities. Examples of this diversity in specificity (including the lectin notation, name, and source) include the following: *N*-acetylglucosamine (LEA, *Lycopersicon esculentum*, tomato), *N*-acetyl galactosamine (PLA, *Phaseolis limensis*, lima bean), galactose (RCL, *Ricinus communis*, castor bean), fucose (LTA, *Lotus tetragonolobus*, asparagus pea), glucose/mannose (Con A, *Canavalia eniformis*, jack bean) and mannose (NPA, *Narcissus pseudonarcissus*, daffodil) (*20*).

The group of lectins defined as the monocotyledonous mannose-specific lectins (MSLs) are unique in that they display an almost exclusive specificity to mannose containing oligo- and polysaccharides and within this group there are distinct preferences for terminal and internal linkages and for different types of glycosidic linkages (α1−2, −3, −4, and −6) (*20*−*22*).

This unique specificity has been exploited directly in the field of therapeutics and we have previously reported the efficacy of NPA at inhibiting the infection of human and feline immunodeficiency viruses (HIV and FIV, respectively) in vitro (*22*−*24*).

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Table 1. Minimal Concentration Required for Agglutination of the Lectins Isolated from Different Species of *Narcissus*[a]

species	haemagglutination	species	haemagglutination
NPA	++	*N. munoz-garmendiae*	+
N. abcissus	++	*N. obesus*	++
N. assoanus	+++	*N. pallidiflorus*	+
N. baeticus	++	*N. pallidulus*	+
N. bujei	++++	*N. panizziarus*	++++
N. bulbocodium	+++	*N. perez-chiscanei*	++
N. cantabricus	++	*N. poeticus*	++
N. confusus	++++	*N. primigenius*	++
N. dubius	++	*N. sussana*	++++
N. eugeniae	++	*N. tortifolius*	++
N. fernandesi	+	*N. tortuosus*	+
N. leginensis	++	*N. triandrus*	++
N. leonensis	++	*N. triaundrus*	+++
N. moschatus	++++	*N. vasconicus*	+

[a] Haemagglutination: +, >NPA (3.1 μg/mL); ++, =NPA (3.1 μg/mL); +++, 0.78 μg/mL; ++++, 0.39 μg/mL. NPA, *Narcissus pseudonarcissus*, commercial lectin preparation.

Nevertheless, the use of an MSL as a therapeutic, or as the basis for one, requires that there be only one isolectin and that it is present in significant amounts. MSLs are known to exhibit a range of isolectins *in planta* (*25*) but the variation within species has been poorly researched. To address this, we chose to study the variability of the MSLs within a group of *Narcissus* species.

MATERIALS AND METHODS

All materials and reagents were purchased from Merck UK Ltd. unless indicated otherwise. Twenty seven species of *Narcissus*, from different sites within Spain, were collected in the resting stage and used for lectin extraction. Bulbs at this stage were chosen because this is believed to be the period of optimal lectin concentration (*20*).

Lectin Isolation. The extraction procedure followed was that described by Van Damme et al. (*26*). Briefly, plant material was rapidly chopped into small pieces and homogenized in 1 M $(NH_4)_2SO_4$ (10 mL [g fresh wt.]$^{-1}$). The homogenate was then centrifuged (25000*g* for 30 min), and the supernatant was frozen overnight at −80 °C. Once thawed the homogenate was recentrifuged as before to remove any precipitate. The clear supernatant was applied to a mannose-agarose column (1.5 × 20 cm) preequilibrated with 1 M $(NH_4)_2SO_4$. Three column volumes of 1 M $(NH_4)_2SO_4$ were run through to remove unbound material. Bound (MSL) material was eluted with 20 mM 1,2-diaminopropane. Eluate was monitored at 280 nm. The eluate was concentrated to 5 mL by rotary evaporation (<40 °C), made up to 1 M $(NH_4)_2SO_4$, and the concentrate was applied to a Phenyl-Sepharose column (1.5 × 20 cm) preequilibrated with 1 M $(NH_4)_2SO_4$. Two column volumes of 1 M $(NH_4)_2SO_4$ were run through the column to remove phenolics, and the lectin was desorbed by elution with d·H_2O. Eluate was monitored at 280 nm and tested for protein using the BioRad Protein Assay (BioRad, UK). The combined protein eluate was freeze-dried.

Haemagglutination Assay. Haemagglutination assays were carried out using 96-well U-bottomed microplates (Corning Costar, UK). Freeze-dried lectins were reconstituted at 2 mg/mL; 50 μL was added to a well and serially diluted to 100 μL with phosphate-buffered saline (PBS, 0.1 M phosphate, 0.7% NaCl, pH 7.2) across the plate. Rabbit erythrocytes (20 μL) which had previously been washed and diluted in PBS at a ratio of 1:10, were then added to each well and incubated at room temperature (20 °C) for approximately 3 h. Agglutination was detected visually; agglutinated cells appeared as a suspension while nonagglutinated cells settled at the bottom of the well and appeared as a precipitate.

Ion-Exchange Chromatography. Isolectins were separated by ion-exchange chromatography using a G-250 fast protein liquid chromatography (FPLC; Amersham-Pharmacia, Upsala, Sweden) system equipped with a Mono-Q anion exchange column. MSL (~40 μg) was loaded on to the column which had been preequilibrated with 20 mM Tris−HCl, pH 8.0. Two column volumes of this buffer were allowed to run through the column before the isolectins were eluted using a linear gradient (30 mL total volume) from 0 to 0.4 M NaCl in buffer at 1 mL min^{-1}. Protein elution was monitored by absorbance at 280 nm.

Isoelectric Focusing (IEF) and Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Isoelectric focusing was performed using a 2117 multiphor II electrophoresis unit (Amersham-Pharmacia). Acrylamide gels (5% w/v) containing ampholytes for a pH 4.5−6 gradient were used. Focusing was carried out for 2.5 h at 2000 V, 12.5 mA, and 12.5 W at 5 °C with 0.1 M alanine and glutamic acid in 0.5 M H_3PO_4 as the cathode and anode solutions, respectively.

Once the run was complete, the pH gradient was determined using a surface pH electrode. A standard curve was established for pH versus band position and this was used to establish absolute MSL isoelectic points (pIs). The gels were fixed in an excess of fixative (30% (v/v) methanol: 10% (w/v) trichloroacetic acid: 3.5% (w/v) sulfosalicylic acid) for 1 h. Prior to silver staining, the ampholytes were removed by several washes of a solution containing 30% methanol/12% trichloroacetic acid over a 48 h period. The gels were then silver stained as described by Dunbar (*6*)

SDS−PAGE was performed essentially as described by Laemmli (*27*) using a Mini Protean II slab gels system (Bio-Rad, UK).

RESULTS AND DISCUSSION

Haemagglutination. The overall recovery of affinity purified lectin was approximately the same for all species at about 2 mg (g fresh wt bulb tissue)$^{-1}$. High concentrations of lectin have previously been reported in *N. pseudonarcissus* (daffodils; up to 15% of the total protein) and bulbs of other members of the *Amaryllidaceae* during the resting period (*25, 26*). In these studies, however, cultivated rather than wild species (and varieties) were used and this may account for the relative reduced levels of total MSL.

Haemagglutination has long been known to be a property of lectins (*28, 29*). Indeed, their ability to selectively agglutinate erythrocytes of a particular human blood group within the ABO system has been known for some time (*30*). Although not active against human erythrocytes, MSLs are known to agglutinate rabbit erythrocytes (*25, 26*).

The majority of the purified MSLs tested displayed similar specific agglutination activities to the commercially available NPA (Sigma-Aldrich, UK) with a minimal concentration required for agglutination of 3.1 μg mL^{-1} (**Table 1**). However, the MSLs from *N. assoanus* (0.78 μg mL^{-1}), *N. bujei* (0.39 μg mL^{-1}), *N. bulbocodium* (0.78 μg mL^{-1}), *N. confusus* (0.39 μg

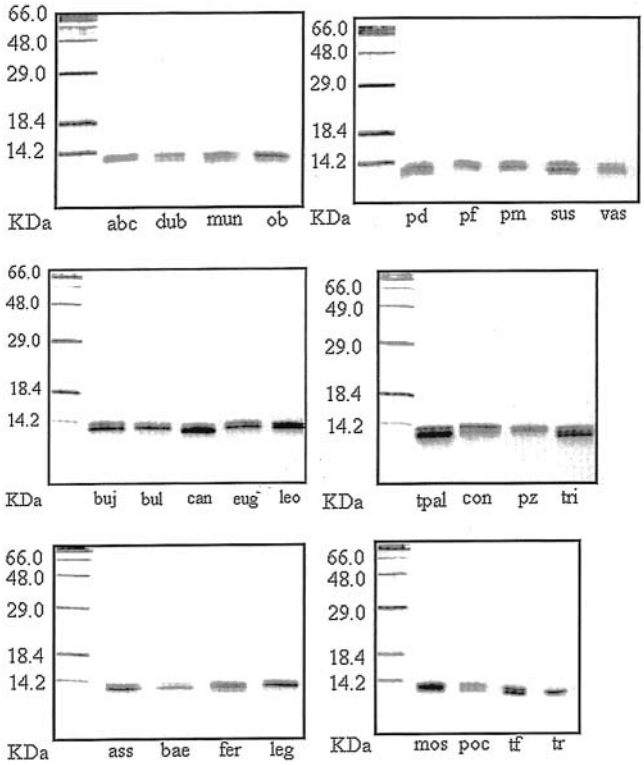


Figure 1. SDS–PAGE of purified lectins from *N. triandrus ssp pallidulus* (tpal), *N confusus* (con), *N. triandrus* (tri), *N. bujei* (buj), *N. bulbocodium* (bul), *N. cantabricus* (can), *N. eugeniae* (eug), *N. leonensis* (leo), *N. assoanus* (ass), *N. baeticus* (bae), *N. fernandesi* (fer), *N. legionensis* (leg), *N. moschatus* (mos), *N. poeticus* (poe), *N. tortifolius* (tor), *N. tortuosus* (tr), *N. abcissus* (ab), *N. dubius* (dub) *N. munozii-garmendiae* (mun), *N. obesus* (ob), *N. pallidulus* (pd), *N. pallidiflorus* (pf), *N. primigenius* (pm), *N. sussanae* (sus), and *N. vasconicus* (vas).

mL^{-1}), *N. moschatus* (0.39 μg mL^{-1}), *N. susannae* (0.39 μg mL^{-1}), and *N. triandus* (0.78 μg mL^{-1}) displayed superior haemagglutination activity.

It has previously been shown that rabbit erythrocytes from different stocks can exhibit variation in agglutination sensitivity but these assays used the same stock throughout. In addition, the inclusion of a commercially available MSL (NPA) as a positive control means that these results are directly comparable to those of other studies employing different erythrocyte stocks.

The haemagglutination activities, and hence the oligosaccharide binding abilities, of these selected *Narcissus* species are greater than those reported for other *Amaryllidaceae*-derived MSLs, such as snowdrop (1.8 μg mL^{-1} (*31*)), tulip (>10 μg mL^{-1} (*32*)), garlic (10−25 μg mL^{-1} (*33*)), ramson (1 μg mL^{-1} (*25*)), and bluebell (15 μg mL^{-1} (*34*)).

Molecular Weight. The use of high concentration acrylamide gels (17%) allowed the separation of the isolectins with similar, but not identical, molecular weights. All the species analyzed yielded two or three bands of around 12.5 kD, the region commonly reported for the monomer of other *Amarylidaceae* MLSs (*19*). The intensities of the bands varied with species (**Figure 1**), indicating that the lectin subunit composition was not uniform across the species. This also meant that their combination to form native lectins will also vary across species. Indeed, previous reports of NPA molecular structure describe the native lectin either as a dimer or trimer when in solution, depending on the method used (*19, 26*), or as a tetramer, in the crystal form and solution, when a single isolectin was used (*35*). In addition, preliminary studies with the MSL from a Chinese *Narcissus* subspecies, *N. tazetta*, have shown that it exists as dimers comprised of ∼13 kDa monomers (*36*).

Ion Exchange Chromatography. A wide and diverse range of chromatographic traces were exhibited by the 27 species studied (**Figure 2**). Even so, when grouped into their appropriate sections according to phenotype (*37*−*40*) remarkable similarities begin to emerge. For example, the MSLs from *N. dubius* and *N. tortifolius*, both in the *Dubii* section, displayed a triumvirate of sharp peaks centered at ∼18 min (A and B, respectively), although in *N. dubius* there is a later eluting poorly resolved peak (C, **Figure 2**).

Similarities were also evident among the MSLs ascribed to the *Bulbocodium* section: *N. bulbocodium*, *N. obesus*, and *N. cantabricus*. They displayed a significant peak (18, 19, and 17.5 min, (D−F) respectively) with 1 or 2 earlier and later eluting peaks. In addition, both *N. bulbocodium* and *N. cantabricus* exhibited a doublet of earlier eluting peaks with similar intensity ratios at 10−12 (G) and 12−14 (H) min, respectively.

Interestingly, the MSL *N. susannae* (section *Bulbobomodes [Edez.]*) exhibits a very distinct chromatogram. According to The Royal Horticultural Society (*40*), this species is actually the product of a cross between *N. cantabricus* and *N. triandus* and this may be reflected in the chromatogram of *N. susannae*. For example, the trio of peaks at 17−19 min (I) in the chromatogram of *N. triandus* is represented in the *N. susannae* trace at 14−18 min (J), their earlier elution time due to an elevated eluent gradient. In addition, the latter two peaks and shoulder in the *N. cantabricus* chromatogram (21−23 min, K) are present in the *N. susannae* chromatogram in an equivalent peak ratio (L).

N. muñozii-garmendiae is, in some classifications (*40*), a subspecies of *N. susannae*, perhaps reflected in the remarkable similarities of their elution patterns. Both displayed a trio of peaks (15−17 (M) and 14−18 min (J), respectively) and a following triplet of peaks at 17.5−20 (*N. muñozii-garmendiae*) and 20−23 min (*N. susannae*), respectively.

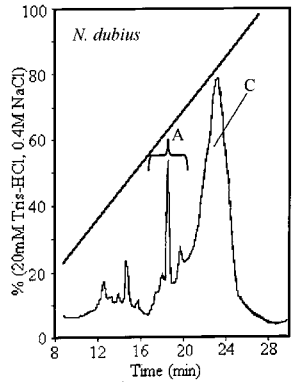
The chromatograms of the majority of the MSLs within the largest section, *Pseudonarcissus*, displayed many common features. These included a central peak at 14−16 min. (depending on the start of the elution gradient) with, in general, one preceding peak and two later-eluting peaks. The absolute peak heights varied from species to species, but in general the elution pattern was maintained. In some cases, the pattern was easily discernible and well resolved (*N. baeticus* (O), *N. leonensis* (P), and *N pseudonarcissus* (Q)). However, in selected cases (*N. tortuosus* and *N. vasconicus*) the resolution remained poor despite our best efforts, but there was still, arguably, the representative trio of peaks present.

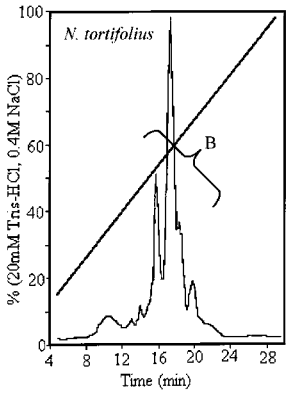
The commonality of the patterns within sections means that "isolectin typing" may be used as an aid to species classification or to confirm prior classification on the basis of phenotype.

Isoelectric Focusing. The MSLs from *N. pseudonarcissus* (NPA) and *N. confusus* (NCA) and fractions from the ion exchange chromatogram of the latter were the subject of isoelectric focusing. A host of bands of varying intensities became apparent following silver staining (**Figure 3**), with even apparent single peaks (I and II) from the IE−FPLC producing many bands. This suggests a further complexity to the native lectins, as they can be composed of monomers differing in both molecular weight and pI.

N. pseudonarcissus has previously been shown to contain over 50 isolectin polypeptides (*26*). Isolation and analysis of the lectin from different tissues and developmental stages further indicated

Section *Dubii*





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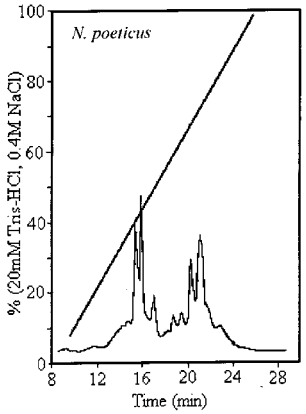
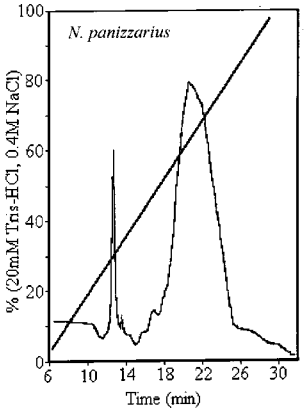
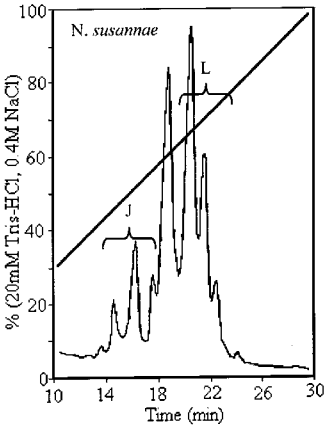


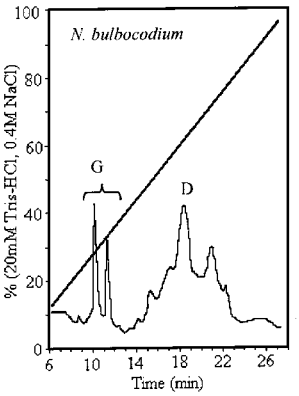
Section *Hermione*

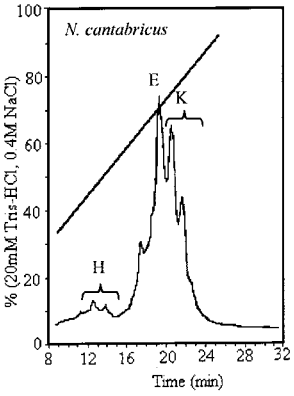


Section *Bulbomodes* (Edez.)

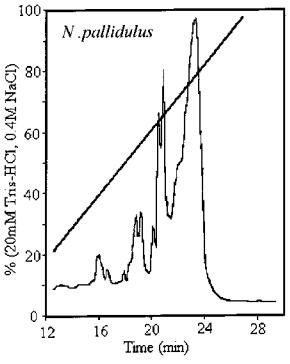


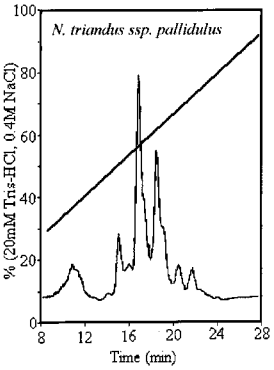
Section Bulbocodium (DC)

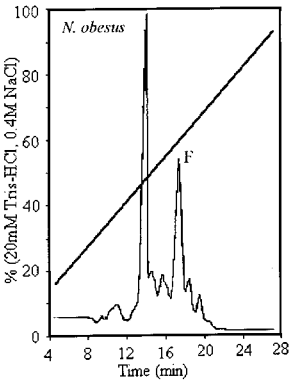


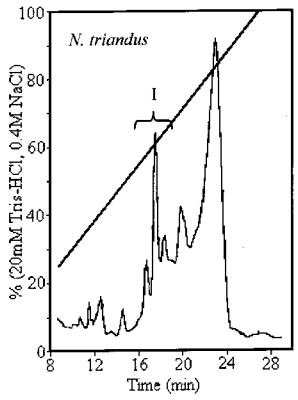


Section *Ganymedes* (Haw. Schult.)









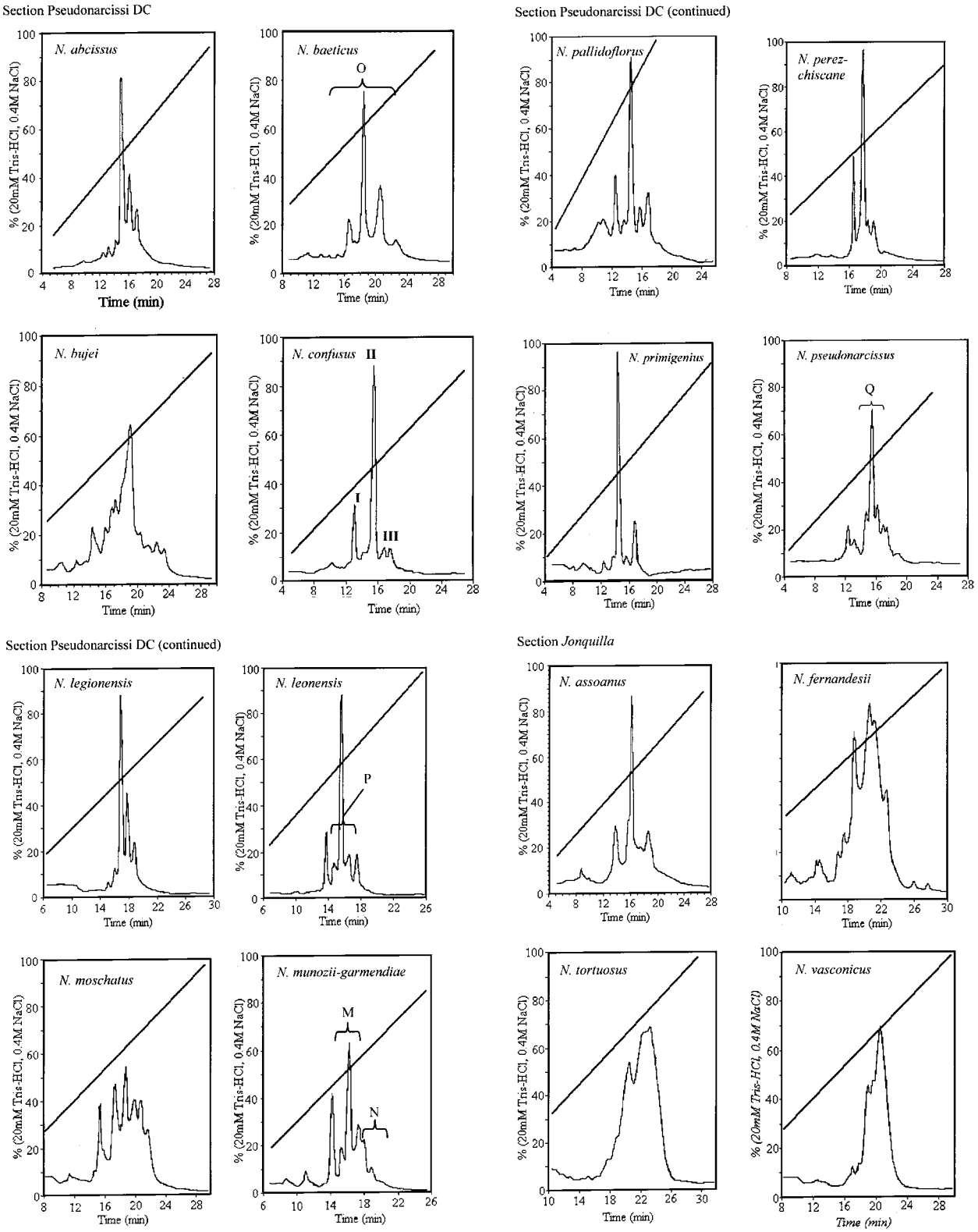


Figure 2. Ion-exchange chromatograms of *Narcissus* MSLs sorted according to phenotypic classification (*37–40*).

that isolectin composition can be both tissue specific and developmentally regulated (*25*).

To date, the degree of isolectin diversity displayed here by the *Narcissus* genus is largely confined to the *Amaryllidacea* and *Alliaceaea*, although with these the greatest degree of diversity rests with the species comprising the *Narcissus* genus. Isolectins have been described in several plant species such as *Phaseolus vulgaris*, *Dolichos biflorus*, *Griffonia simplicifolia*, *Glycine max*, and *Datura stramonium*. For some of these species the origin of the isolectins is understood. For example, *P. vulgaris* agglutinin (PHA) expresses 2 distinct lectin monomer proteins, erythrocyte (E) and lymphocyte (L) reactive subunits, which combine into tetramers to give 5 distinct native lectins: L_4, L_3E, L_2E_2, LE_3, and E_4 (*41*). The presence, absence, and difference in degree of glycosylation can also give rise to the presence of multiple isolectins. Conversely, the isolectins of the *Narcissus* (and selected species within the *Amaryllidacea* and *Alliaceae* families) are derived from nonglycosylated monomers

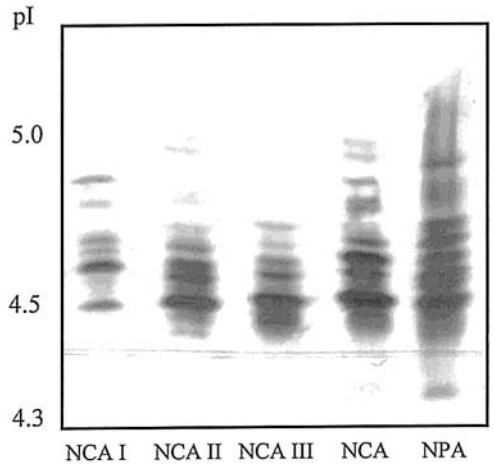


Figure 3. Isoelectric focusing gel of the MSLs from *N. pseodonarcissus* (NPA) and *N. confusus* (NCA) and fractions (I, II, and III, see **Figure 2**) from the ion exchange chromatogram of the latter.

with virtually identical molecular weights. The source of diversity for these isolectins can be 2-fold: posttranslational proteolytic trimming and/or encoding of the lectin monomers by a multiple gene family. Indeed, it has been shown that *Narcissus*, and the attendant subspecies, are polyploidy (*41*) and exist almost exclusively as tetraploids (although there are examples of triploids). This means that there are four copies of each chromosome and therefore each (lectin) gene. Minor evolutionary mutation events, such as deletion, translocation, and inversion, would lead to the expression of lectin subunits with very slightly different amino acid compositions. The implications of this on a tertraploid system would mean that a potential broad range of very similar proteins may be expressed, the situation evident in the *Narcissus* species.

CONCLUSION

The MSLs of the *Narcissii* exhibit only slight variation in their monomer subunit with respect to molecular weight values of, or around, 12.5 kD. Nevertheless, a significant degree of variation exists with regard to the isoelectric point, pI, which has resulted in the existence, in vivo, of many distinct native lectins.

Within the limited group of *Narcissus* species studied here, haemagglutination, and therefore oligo/polysaccharide binding abilities, were at least equal to, and in selected cases up to more than four times greater than, that of other previously reported MSLs from snowdrop (*Galanthus nivalis*, GNA) and ramson (*Allium ursinum*, AUA) (*31*, *25*). Interestingly, GNA has been the MSL of choice for transformation studies into plant insect resistance (*43*−*46*). Given the superior binding abilities of the commercial (*22*) and wild *Narcissus* species demonstrated here it would seem that they would be a more logical choice for any further studies.

ACKNOWLEDGMENT

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