JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

NsLTP1 and NsLTP2 Isoforms in Soft Wheat (*Triticum aestivum* Cv. Centauro) and Farro (*Triticum dicoccon* Schrank) Bran

ANTONELLA CAPOCCHI,*,† DEBORA FONTANINI,† VERA MUCCILLI,§ VINCENZO CUNSOLO,§ FRANCO SAVIOZZI,† ROSARIA SALETTI,§ ROBERTO LORENZI,† SALVATORE FOTI,§ AND LUCIANO GALLESCHI†

Department of Botanical Sciences, University of Pisa, via L. Ghini 5, 56126 Pisa, Italy, and Department of Chemical Sciences, University of Catania, Viale A. Doria 6, 95125 Catania, Italy

Isoforms of nonspecific lipid-transfer protein 1 (nsLTP1) and nonspecific lipid-transfer protein 2 (nsLTP2) were investigated in bran tissues isolated from caryopses of two cereal crops quite relevant for the Italian market, the cultivar Centauro of soft wheat (*Triticum aestivum*) and Italian emmer or farro (*Triticum dicoccon* Schrank). By sequential separation of the bran extracts on cation-exchange and gel filtration chromatographies, fractions containing only proteins belonging to the nsLTP1 and nsLTP2 classes were obtained. The proteins were roughly identified by SDS-PAGE and by immunoreactions in Western blotting experiments. By MALDI-MS and RP-HPLC/ESI-MS analyses we were able to show the presence of several LTP1 and LTP2 isoforms in the investigated species. Bioinformatic searches based on the determined M_r indicated that (i) two nsLTP1s already identified in *T. aestivum* have M_r and number of Cys residues identical to that of a 9.6 kDa protein present both in soft wheat cv. Centauro and in farro; (ii) two isoforms of nsLTP2 detected in *T. aestivum* have the same M_r and number of Cys residues of two 7 kDa proteins found in Centauro; and (iii) a nsLTP1 detected in *Ambrosia artemisiifolia* has M_r and number of Cys residues coincident to that of a 9.9 kDa protein found both in soft wheat cv. Centauro and in farro.

KEYWORDS: Farro; isoform; MALDI-MS; plant lipid-transfer protein; RP-HPLC/ESI-MS; *Triticum aestivum*; *Triticum dicoccon*

INTRODUCTION

Plant nonspecific lipid transfer proteins (nsLTPs) are a widely distributed superfamily of related proteins (*1*) that bind and transfer a broad range of phospholipids between membranes, in vitro (*2*). Their biological function in plants is still unclear; they may be involved in the formation of the cuticle layer (*3*), in somatic embryogenesis (*4*), and in plant responses to abiotic (drought and salinity) and biotic stresses (*5*−*7*). Recent works, demonstrating that elicitins and nsLTPs share a similar molecular structure and the same biological receptors, give a new perspective in understanding the role played by nsLTPs, mainly in the early recognition of plant pathogens (*8*, *9*).

Two main families of plant nsLTPs are described: the 9 kDa nsLTP1 and the 7 kDa nsLTP2. NsLTP1 and nsLTP2 are cysteine-rich proteins, with compact structures consisting of four α-helices connected by three loops and a C-terminal segment enclosing a hydrophobic cavity. Four strictly conserved disulfide bonds interconnect the secondary structure elements (*2*). Plant nsLTPs are encoded by a small multigene family in a variety of plant species (*1*). Different nsLTP isoforms can have different functions; four isoforms isolated from castor bean seedlings are differentially expressed in a tissue-specific manner (*10*). Two different nsLTPs have been found in tobacco, one of which is expressed mainly in the tapetal cells (*11*), whereas the other is expressed in all aerial portions of the plant (*12*). Chemicals such as inorganic salts, salycilic acid, and ABA, as well as pathogen infections and environmental stresses, all modulate nsLTPs expression, depending on tissue and nsLTP gene classes, suggesting a complex regulation of these genes (*13, 14*).

NsLTPs are ubiquitous in the plant kingdom and are especially abundant in higher plants, where they can represent as much as 4% of the total soluble proteins (*1*). They are localized mainly in the aerial portions of vegetative and reproductive tissues. Immunocytochemical studies carried out in maize (*Zea mays*) (*15*), castor bean (*Ricinus communis*) (*16*), land cress (*Arabidopsis thaliana*) (*17*), and broccoli (*Brassica oleracea*) (*18*) have demonstrated the presence of nsLTPs in the cell wall of leaves, petioles, stems, flowers, siliqua, and vascular bundles. NsLTP1 and nsLTP2 have also been isolated from seeds of several species, such as barley (*Hordeum vulgare*) (*19*, *20*), rice (*Oryza sativa*) (*21*, *22*), wheat (*Triticum aestivum*) (*23*, *24*), maize (*25*), and amaranth (*Amaranthus hypocondriacus*) (*26*).

* Author to whom correspondence should be addressed (telephone +39 050 2215360; fax +39 050 2215380; e-mail capocchi@dsb.unipi.it).
† University of Pisa.
§ University of Catania.

10.1021/jf0580465 CCC: $30.25
Published on Web 09/10/2005

The subcellular localization of nsLTP1 in mature wheat seeds, as determined with polyclonal anti-nsLTP1 antibodies, showed specific labeling in the aleurone grains. Neither the cell wall, the nucleus, nor the cytoplasm was labeled (*27*). In *Vigna unguiculata* seeds, immunofluorescence assays indicated that nsLTPs were localized in the cell walls and in intracellular compartments such as the protein storage vacuoles of cotyledons and embryonic axes (*28*).

The majority of nsLTPs are secreted proteins; however, it cannot be excluded that some nsLTP isoforms are addressed to cytosolic compartments; for example, nsLTPs from castor bean appeared to be partly located (13%) within the glyoxysome (*16*), where they would function as acyl-CoA carriers (*29*).

In the present paper we investigate the presence of nsLTP1 and nsLTP2 isoforms in *T. aestivum* L. cv. Centauro and in *Triticum dicoccon* Schrank seeds. *T. aestivum* cv. Centauro is a soft wheat broadly cultivated in Italy, and *T. dicoccon* is a hulled wheat, whose cultivation is limited to a rather restricted area of the Central Apennines. Cultivated hulled wheats, known in Italy as farro (emmer), are among the most ancient Triticeae grown by humankind. However, they have been progressively replaced with high-yielding varieties of naked wheats. It was only in the past 15 years that increased attention to natural food products and to organic agricultural practices has brought a renewed attention to this forgotten crop.

Our interest in these proteins was driven by the identification of nsLTPs as major allergenes in fruits from Rosaceae plants, such as peach, apple, and apricot (*30*). Asero et al. (*31*) reported on allergies toward a broad range of nonfruit vegetable foods caused by nsLTPs. The wide distribution of nsLTPs in plants suggests a potential role of these proteins as plant panallergens that are capable of inducing severe and nearly fatal responses in allergic individuals (*32*). Nevertheless, food products, in which cereal whole flours are used, are not major inducers of nsLTP-based allergic reactions. It is not clear yet, if wheat, rye, and barley nsLTPs are altered during food processing or if they are intrinsically low allergenic proteins. Overall, these characteristics, combined with their resistance to proteases, stress their clinical importance (*33*).

MATERIALS AND METHODS

Plant Materials. Seeds of the soft wheat *T. aestivum* cv. Centauro and seeds of *T. dicoccon* (emmer; Italian farro) were purchased from the Società Produttori Sementi S.p.A. (Bologna, Italy) and from the Consorzio Produttori Farro della Garfagnana (Piazza al Serchio, Lucca, Italy), respectively. The seeds were ground in a break roller-mill (Labormill 4RB, Bona, Monza, Italy) that allowed for the separation of flour, bran and—in the case of farro—hulls. The farro bran obtained was further cleaned from residual flour and hulls by sieving through a 6−18 mesh with the AS200 analytical sieve shaker (Retsch GmbH & Co. KG, Haan, Germany). The bran samples were frozen and stored at −20 °C until used.

Extraction of NsLTPs. One hundred grams of each bran was extracted overnight at 4 °C, by gentle stirring, with 1 L of distilled water. After the extraction, the slurry was squeezed through four layers of cheesecloth and centrifuged at 6500 rpm for 10 min at 4 °C. The supernatant was brought to 80% saturation with solid ammonium sulfate and stirred for 2 h at 4 °C. After centrifugation at 6500 rpm for 10 min, the precipitate was suspended in 10 mM ammonium acetate (pH 6.8) and dialyzed overnight at 4 °C (3.5 kDa dialysis tubing cutoff) against the ammonium acetate buffer. The dialyzed extract was centrifuged at 15000 rpm for 10 min at 4 °C. The supernatant was loaded on a CM-cellulose column (20 × 2.5 cm; Sigma Chemical Co., St. Louis, MO) that was equilibrated with 10 mM ammonium acetate (pH 6.8). After the nonbound material had been removed by washing with equilibration buffer, the column was eluted at a flow rate of 1 mL/min with a linear gradient from 10 to 250 mM of ammonium acetate (pH 6.8) buffer. The collected fractions were analyzed by SDS-PAGE on 15% acrylamide (w/v) minigels, at 15 mA constant current (*34*). After the run, the minigels were fixed and stained according to the method of Koenig et al. (*35*). The protein molecular weights were estimated with low molecular weight markers (BDH, Poole, U.K.) added with insulin β-chain (0.35 mg/mL final) from bovine pancreas (Sigma Chemical Co., St. Louis, MO). The fractions containing proteins with molecular mass between 12.3 and 5.7 kDa were pooled and brought to 80% saturation with solid ammonium sulfate. The ammonium sulfate precipitate was resuspended in 1.5 mL of 10 mM ammonium acetate buffer (pH 6.8) and loaded on a (2 × 77 cm) Sephadex G-50 column (Pharmacia Biotech, Uppsala, Sweden), equilibrated with 10 mM ammonium acetate buffer (pH 6.8). Elution was carried out at a flow rate of 1 mL/min, and the eluted fractions were analyzed by 15% SDS-PAGE. The apparent molecular weights of the protein bands were estimated by the gel analysis software Sigma Gel (Jandel Corp., San Rafael, CA).

Fractions exhibiting bands with molecular mass between 12.3 and 5.7 kDa were pooled and freeze-dried.

Western Blotting. The pooled fractions from Sephadex G-50 were subjected to 15% SDS-PAGE; duplicates of the gels were electrotransferred overnight at 60 V onto a 0.45 μm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) by tank blotting (Hoefer Pharmacia Biotechnology Inc., San Francisco, CA). The electrode buffer contained 0.025 M Tris, 0.192 M glycine, and 20% methanol (v/v), pH 8.3. The blotted membranes were washed 2 × 15 min with TBS (50 mM Tris and 150 mM NaCl, pH 7.5), incubated for 1 h with 3% skimmed milk in TBS, and shaken for 2 h in the presence of 20 mL of anti-barley nsLTP1 serum (a kind gift from Dr. B. L. Jones, Barley and Malt Laboratory, USDA-ARS, CCRU, Madison, WI), which had been diluted 1000-fold with 3% skimmed milk in TBS. The nitrocellulose membranes were washed 2 × 15 min with TBS and then shaken for 1 h with the secondary antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase, diluted 1000-fold with 3% skimmed milk in TBS. The membranes were washed 2 × 15 min with TBS, and the peroxidase activity was visualized with 0.05% 4-chloro-1-naphthol (w/v), 17% methanol (v/v), and 0.015% hydrogen peroxide (v/v). The reaction development was stopped by washing with distilled water.

Alkylation with Iodoacetamide. The sample was dissolved in 1 M Tris-HCl, pH 8.5 (1 μg/μL). Dithiothreitol (DTT; Sigma) (10-fold molar excess over the disulfide bonds) was added, and the reduction was carried out at room temperature for 3 h in nitrogen atmosphere. At the end of the reduction, iodoacetamide (IAA; Sigma) was added to the mixture at a molar IAA/DTT ratio of 2:1, and the alkylation was performed for 1 h in the dark, at room temperature. The reaction was stopped by cooling in liquid nitrogen, and the mixture was immediately freeze-dried.

RP-HPLC/ESI-MS. HPLC-grade H_2O and CH_3CN were provided by Lab-Scan (Dublin, Ireland). One hundred gram aliquots of the extracts were dissolved (1 μg/μL) in CH_3CN 0.05% trifluoroacetic acid (TFA; Sigma)/H_2O 0.05% TFA (20:80, v/v), filtered through a 0.45 μm Micro-spin filter (Lida, Kenosha, WI), and subsequently analyzed by RP-HPLC and mass spectrometry.

On-line RP-HPLC/ESI-MS analysis was performed by loading 30 μL of sample solution, corresponding to 30 μg, onto a Vydac C4 narrow-bore column (2.1 × 250 mm, 300 Å pore size, 5 μm particle size) coupled with an electrospray ion source of a DECA ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Proteins were eluted at 50 °C with a linear gradient of CH_3CN 0.05% TFA/H_2O 0.05% TFA from 20 to 60% in 60 min at a flow rate of 200 μL/min. Electrospray mass spectra were acquired from *m*/*z* 600 to 2000 using the following electrospray ion source parameters: capillary temperature, 200 °C; capillary voltage, 46 V; gas flow rate, 80 ua (corresponding to 1.2 L/min).

MALDI-TOF MS. MALDI mass spectra were acquired using a Voyager DE-PRO time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a UV nitrogen laser (337 nm). Spectra were obtained in positive linear ion mode and were averaged from ∼150 laser shots to improve the signal-to-noise level. Sample preparation was performed following two different procedures. In one

procedure, the sample was prepared according to the dried droplet method (*36*), using sinapinic acid (SA) as matrix. The matrix solution was prepared by dissolving SA in CH_3CN/0.1% TFA (60:40, v/v) at a concentration of 10 μg/μL. Alternatively, the protein mixture was subjected to a micropurification (desalting/concentration) with a homemade 5 mm nanocolumn packed with C18 resin (POROS R2) (Applied Biosystems) in a constricted GELoader tip (Eppendorf Scientific, Westbury, NY), according to the method of Gobom et al. (*37*). For analyses by MALDI-MS, the proteins were eluted with 0.8 μL of matrix and deposited directly onto the MALDI target.

Mass assignments were made using insulin (5734 Da) and cytochrome *c* (12360 Da) as external standards.

Bioinformatic Search. M_r values of nsLTPs, determined by mass spectrometry for the protein components of soft wheat pool G-50 and farro pools I G-50 and II G-50, were used to search the NCBInr database using the Molecular Weight Search field of the Entrez Proteins database (PubMed server) (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein&itool=toolbar).

To carry out the search, the experimentally determined M_r was incremented by 8 Da to take into account the reduced form of the eight cysteine residues present in the sequence. The search was limited by organism (*Triticum* or *Viridiplantae*) and used a range of molecular mass of ±3 Da with respect to the experimental value, according to the uncertainty of the measure.

RESULTS

Partial Purification and Western Blotting of Soft Wheat and Farro Extracts. The soft wheat bran extract separated by cation-exchange chromatography showed, on SDS-PAGE gels, proteins of 9 and 7 kDa that eluted with the ammonium acetate gradient between 170 and 250 mM (**Figure 1A**). These fractions were collected and pooled (Centauro pool CM) for further purification by gel filtration chromatography. The SDS-PAGE analyses of the farro bran extract after cation-exchange chromatography indicated that two areas of the chromatogram contained proteins of interest. The first area included proteins of 9 and 7 kDa that eluted with the ammonium acetate gradient between 170 and 220 mM; the second area included proteins of 9 kDa eluting between 220 and 250 mM with the ammonium acetate gradient (**Figure 2A**). The fractions corresponding to those comprising the two chromatographic areas were collected, pooled, and named "farro pool I CM" and "farro pool II CM", respectively. The pools obtained from the cation-exchange chromatographies were further purified by gel filtration chromatography.

Chromatography on the Sephadex G-50 column of the Centauro pool CM separated the sample into two large peaks (**Figure 1B**). The SDS-PAGE analysis of these fractions showed that the first eluting peak contained protein bands with molecular masses ranging from 66.25 and 17.2 kDa, whereas the second peak contained low molecular mass polypeptides (about 9 and 7 kDa).

When farro pools I CM and II CM were separated by gel filtration chromatography (**Figure 2B**), they both split into a large peak containing relatively high molecular mass polypeptides (ranging from 42.7 to 17.2 kDa on SDS-PAGE) and a small peak. The farro pool I CM small peak contained almost exclusively 9 and 7 kDa proteins, whereas the farro pool II CM small peak contained only 9 kDa proteins. The fractions of interest from both gel filtrations were collected and pooled as indicated in **Figures 1B** and **2B**.

The SDS-PAGE analyses of the G-50 pools (one from the soft wheat sample and two from the farro sample) protein patterns are shown in **Figure 3A**. Centauro pool G-50 showed two protein bands with molecular masses of approximately 9 kDa and 6.8 kDa (**Figure 3A**, lane 1); these protein bands were also present in farro pool I G-50 (**Figure 3A**, lane 2). Farro pool I G-50 also showed two contaminants with higher molecular masses (28 and 18 kDa). Farro pool II G-50 mainly comprised a protein band with an apparent molecular mass of 8.8 kDa. Duplicate gels were run for Western blotting experiments. Incubating the membrane in the presence of anti-nsLTP1 serum gave a strong, positive immunoreaction for the Centauro and farro pool I 9 kDa proteins and for the farro pool II 8.8 kDa protein; a less intense reaction was observed between the antibodies and the 18 kDa protein in farro pool I (**Figure 3B**, lanes 1−3).

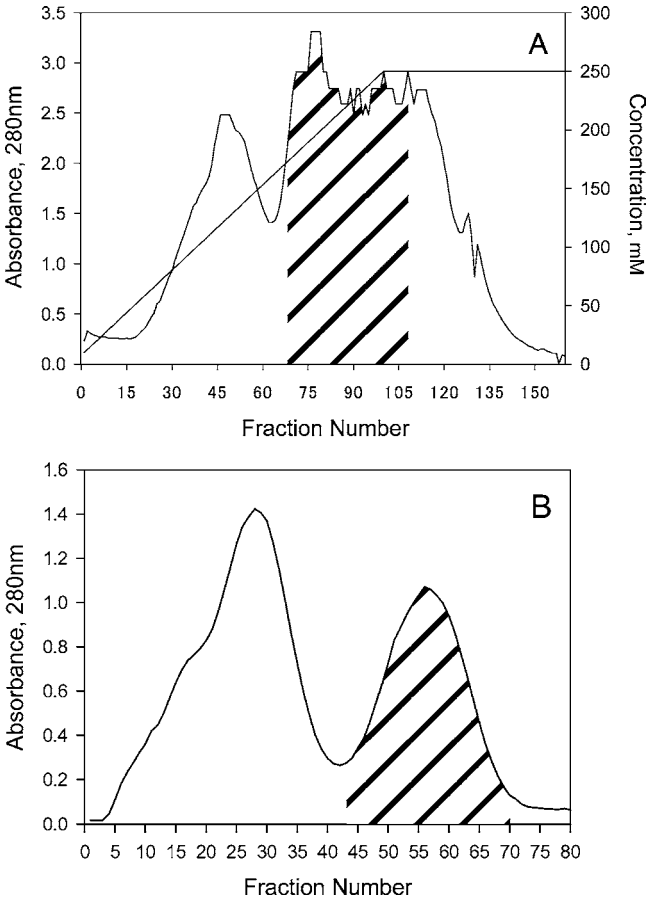

Figure 1. Cation-exchange and gel filtration chromatographies of the soft wheat extract: (**A**) CM-cellulose chromatography. Protein elution was monitored at 280 nm; the shaded area comprises those fractions that were pooled into Centauro pool CM. The elution gradient was plotted on the *y*-secondary axis. (**B**) G-50 chromatography of Centauro pool CM. The shaded area indicates the fractions that were pooled into Centauro pool G-50.

MALDI-TOF Analyses of Soft Wheat and Farro G-50 Pools. MALDI-TOF mass spectra were acquired in the *m*/*z* range 2−15 kDa. The MALDI-TOF mass spectrum of Centauro pool G-50 showed the presence of two protein groups, the first one characterized by a major component at *m*/*z* 6976 and a minor one at *m*/*z* 7042 and the second one characterized by proteins having protonated molecular ions at *m*/*z* 9475, 9612, 9793, 9929, and 10499 (**Figure 4**). Signals corresponding to the doubly charged molecular ions of the 9 kDa proteins were also present in the spectrum in the *m*/*z* 4738−4966 mass range.

The number of cysteine residues present in each protein component was determined by reduction of the protein mixture and alkylation of the free thiol groups, a treatment that results in a molecular mass increase of 57 Da for each free thiol group. From total analysis of the MALDI mass spectrum of the reduced

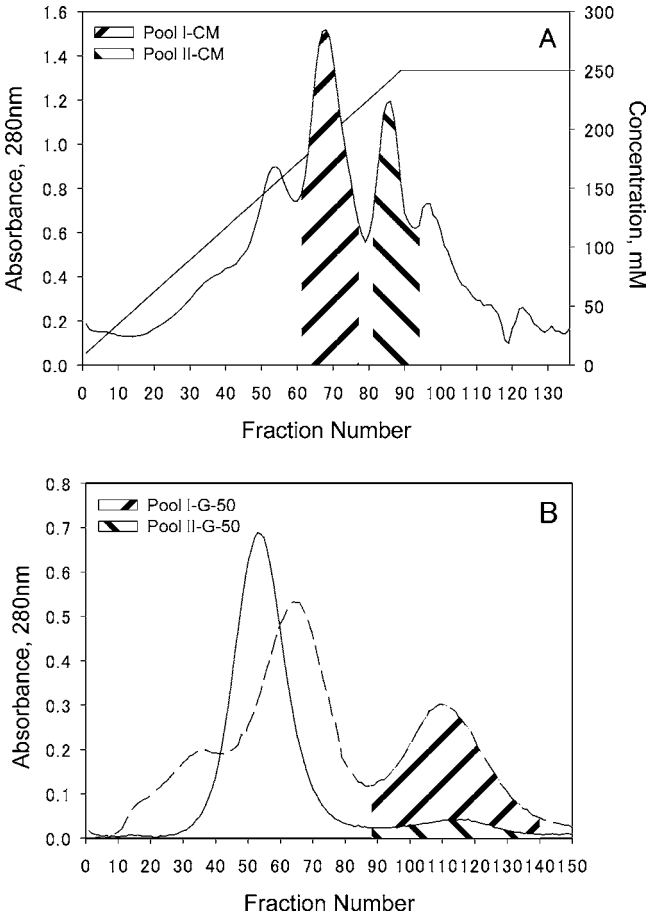

Figure 2. Cation-exchange and gel filtration chromatographies of the farro extract: (**A**) CM-cellulose chromatography. Protein elution was monitored at 280 nm; the shaded area comprises those fractions that were pooled into farro pools I CM and II CM, as depicted in the figure legend. The elution gradient was plotted on the *y*-secondary axis. (**B**) G-50 chromatography of farro pools I CM and II CM. The shaded areas indicate the fractions that were pooled into farro pools I G-50 and II G-50, as depicted in the figure legend.

and alkylated mixture (data not shown), it was possible to correlate six protein components containing eight cysteine residues each (**Table 1**). The most abundant proteins in Centauro pool G-50 had molecular masses of 6975 and 9611 Da.

The MALDI-TOF mass spectrum of farro pool I G-50 (**Figure 5**) showed one group of proteins constituted by a major component at *m*/*z* 7004 and a minor one at *m*/*z* 7323 and a second group of proteins in the *m*/*z* range 9−10 kDa, some of which had molecular masses equal to those of the Centauro pool members (*m*/*z* 9611, 9931, and 10032). The MALDI-TOF mass spectrum of farro pool II G-50 showed one protein at *m*/*z* 5649 and traces of two proteins at *m*/*z* 6998 and 7020. It also showed a set of higher molecular weight proteins with components at *m*/*z* 9599, 9894, 9908, 9987, and 10222 (**Figure 6**).

Total MALDI-TOF mass spectra analyses of the reduced and alkylated farro pool I G-50 and pool II G-50 proteins (data not shown) allowed four protein components to be correlated with eight cysteine residues each in pool I (**Table 2**) and with five protein components with eight cysteine residues each in pool II (**Table 3**). Overall, the MALDI-TOF mass spectra indicated that the most abundant protein components in the farro pools had molecular masses of 7003 and 9610 Da (**Table 2**).

RP-HPLC/ESI-MS On-line of the Soft Wheat and Farro Pools from G-50. Figure 7A shows the chromatograms of the

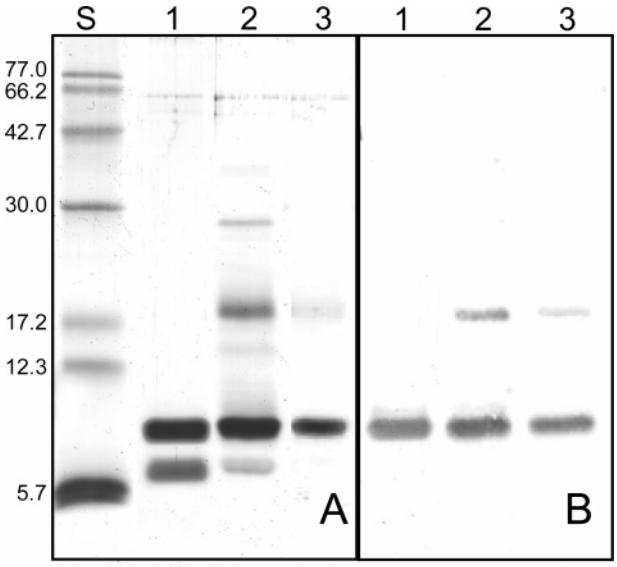

Figure 3. SDS-PAGE and immunoblotting analyses of the soft wheat and farro nsLTP fractions pooled after separation by gel filtration chromatography: (**A**) Coomassie Brilliant Blue staining of the 15% SDS-PAGE; (**B**) after 15% SDS-PAGE, the proteins were electroblotted on nitrocellulose membrane and reacted with antibodies against barley nsLTP1: (lanes 1) soft wheat pool G-50; (lanes 2) farro pool I G-50; (lanes 3) farro pool II G-50. S, molecular weight standard.

soft wheat pool G-50 reconstructed by total ion current (TIC). The data obtained confirmed the presence of the same protein components indicated by the MALDI-TOF analyses (**Table 1**). Deconvolution of multicharged spectra correlated to chromatographic peaks with retention times of 34 and 35 min showed the presence of two proteins with molecular masses (M_r) of 6972 and 7036 Da, respectively. The proteins of 9−10 kDa were detected in a chromatographic area (40−53 min retention time) without defined chromatographic peaks. The reduced/alkylated sample was analyzed under the same conditions (**Figure 7B**). In the chromatographic peak with a retention time of 41 min coeluted two components with M_r of 7436 and 7504 Da, which corresponded to 6972 and 7036 Da reduced and alkylated proteins containing eight cysteine residues each. The most intense peak (with a retention time of 43 min) contained a 10064 Da protein that was correlated to the protein component of 9600 Da plus eight cysteine residues (**Table 1**). A minor peak with a retention time of ∼44 min was due to the elution of a 9932 Da protein, which was correlated to the component of 9468 Da having eight cysteine residues. Finally, a protein of 10388 Da eluted in a chromatographic region at ∼53 min without defined chromatographic peaks. This component corresponded to the 9924 Da protein plus eight cysteine residues.

RP-HPLC/ESI-MS of farro pool I G 50 showed two proteins of 6996 and 7324 Da and thus confirmed the data from the MALDI-TOF analyses. The remaining protein components were present in very low quantities. The chromatogram obtained with the same reduced and alkylated sample showed, at a retention time of 38 min, two proteins with M_r of 7464 and 7808 Da, which corresponded to the reduced and alkylated proteins of 6996 and 7324 Da, containing eight cysteine residues each (**Table 2**). A 10064 Da protein, eluting with a retention time of 43 min, correlated to the component of 9596 Da plus eight cysteine residues.

RP-HPLC/ESI-MS analysis of farro pool II G-50 confirmed the presence of the proteins with M_r of 9596, 9892, and 9908 Da detected by MALDI-MS analysis of the same fraction. The

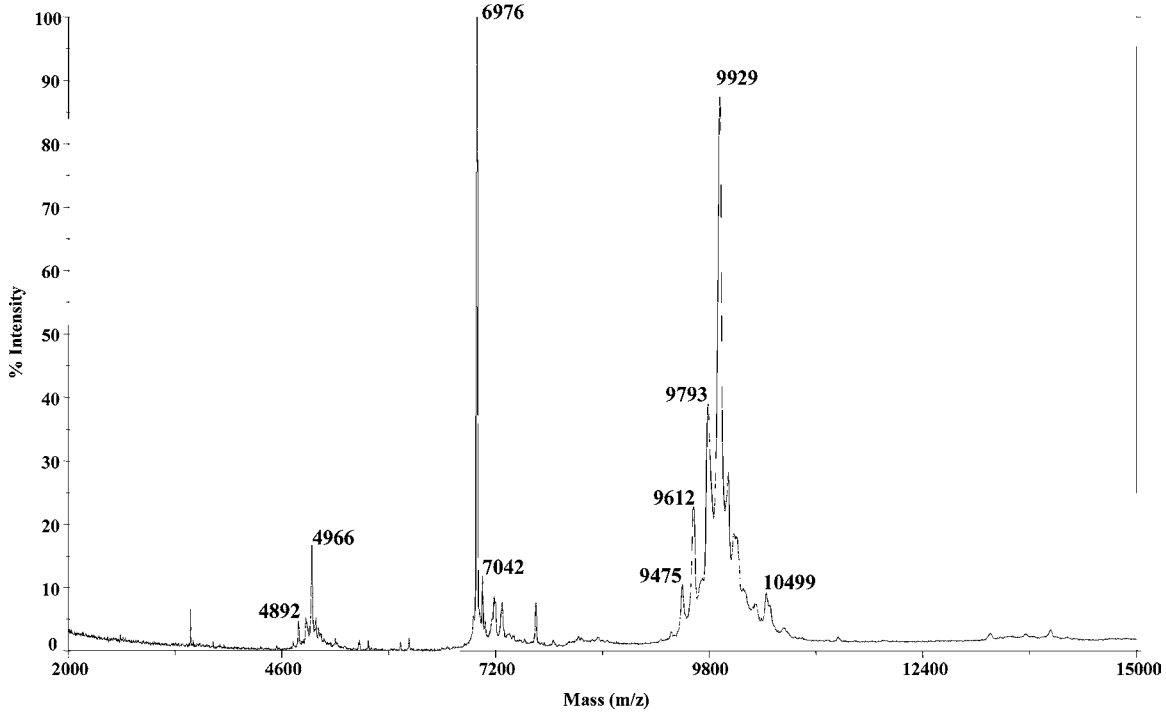

Figure 4. MALDI-TOF mass spectrum of Centauro pool G-50 in the *m*/*z* range 2000–15000. The spectrum was obtained using sinapinic acid as matrix and calibrated externally with insulin and cytochrome *c*.

Table 1. Determination by MALDI-MS and RP-HPLC/ESI-MS Analyses of the M_r and Number of Cys Residues of the Protein Components in Centauro Pool G-50 and Results of Bioinformatic Search

unalkylated proteins exptl M_r		alkylated proteins exptl M_r		
MALDI	ESI	MALDI	ESI	bioinformatic search results
6975	6972	7435 (8.07 Cys)	7436 (8.14 Cys)	P82900 (*T. aestivum*, calcd M_r 6971)
7041	7036	7502 (8.09 Cys)	7504 (8.21 Cys)	P82901 (*T. aestivum*, calcd M_r 7038)
9474	9468	9956 (8.45 Cys)	9932 (8.14 Cys)	
9611	9600	10064 (7.95 Cys)	10064 (8.14 Cys)	CAH04989 (*T. aestivum*, calcd M_r 9600.80) S21757 (*T. aestivum*, calcd M_r 9598.82)
9792	9780	10263 (8.26 Cys)		
9928	9924	10391 (8.10 Cys)	10388 (8.14 Cys)	O04004 (*A. artemisiifolia*, calcd M_r 9921)

chromatogram obtained with the reduced and alkylated farro pool II G-50 indicated, at a 43 min retention time, the presence of the most abundant protein component of the mixture (M_r of 10064 Da), which correlated to the 9596 Da component plus eight cysteine residues (**Table 3**).

DISCUSSION

SDS-PAGE analyses (**Figure 3A**) of the fractions collected after gel filtration chromatography of the soft wheat and farro samples shared two sets of proteins that possibly matched nsLTPs. One set was composed of protein bands with apparent molecular masses of 9 kDa (soft wheat) and 8.8 kDa (farro); also, both samples showed a second protein with an apparent molecular mass of 6.8 kDa. These sets of proteins had a different behavior when analyzed by immunoblotting (**Figure 3B**). In fact, the 9 and 8.8 kDa proteins reacted positively with barley anti-nsLTP1 polyclonal antibodies, whereas the 6.8 kDa proteins did not give any reaction against the same antibodies.

On the basis of these indications, the 6.8 kDa proteins were tentatively identified with nsLTP2. This is consistent with the finding that a wheat kernel 6.9 kDa protein, which was identified with a nsLTP2, did not react against a mixture of barley antibodies that recognized nsLTPs from various species (*38*). Moreover, nsLTP2 sequences show weak similarity (<25%) to the corresponding nsLTP1 (*39*).

The data obtained from MALDI-TOF/MS and RP-HPLC/ESI-MS analyses allowed a more precise determination of the M_r of the protein components in the Centauro pool G-50 and in farro pools I and II G-50 and also aided us in establishing that some of the proteins detected contain eight Cys residue in their sequences as shown in **Tables 1**−**3**. To ascertain whether nsLTPs with the same M_r were already known, the determined M_r values were used to perform a bioinformatic search in the NCBInr database. The results are summarized in **Tables 1**−**3**. As shown in **Table 1**, a nsLTP2 isolated from *T. aestivum* seeds (accession no. P82900) (*40*) has the same M_r and number of Cys residues of the most abundant protein belonging to the nsLTP2 family present in Centauro pool G-50 (6972 Da, ESI-MS). An analogous protein of 6980 Da (accession no. Q9FEK9) was isolated from *T. durum* Desf. (Chandur) seeds. The primary structure of this protein, determined by sequencing fragments released by V8 protease and trypsin proteolysis, evidenced a microheterogeneity at position 58, suggesting that *T. durum* may contain two isoforms of this protein, one of which is identical to that isolated from *T. aestivum* (*24*). The soft wheat protein

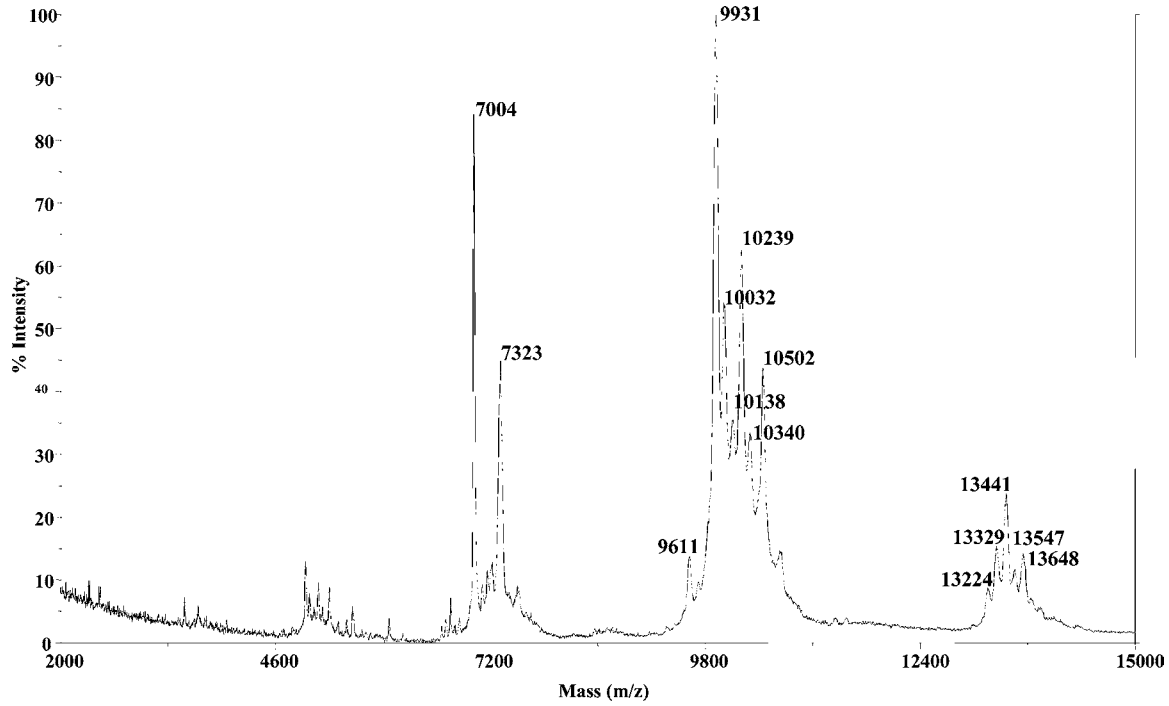

Figure 5. MALDI-TOF mass spectrum of farro pool I G-50 in the *m*/*z* range 2000–15000. The spectrum was obtained using sinapinic acid as matrix and calibrated externally with insulin and cytochrome *c*.

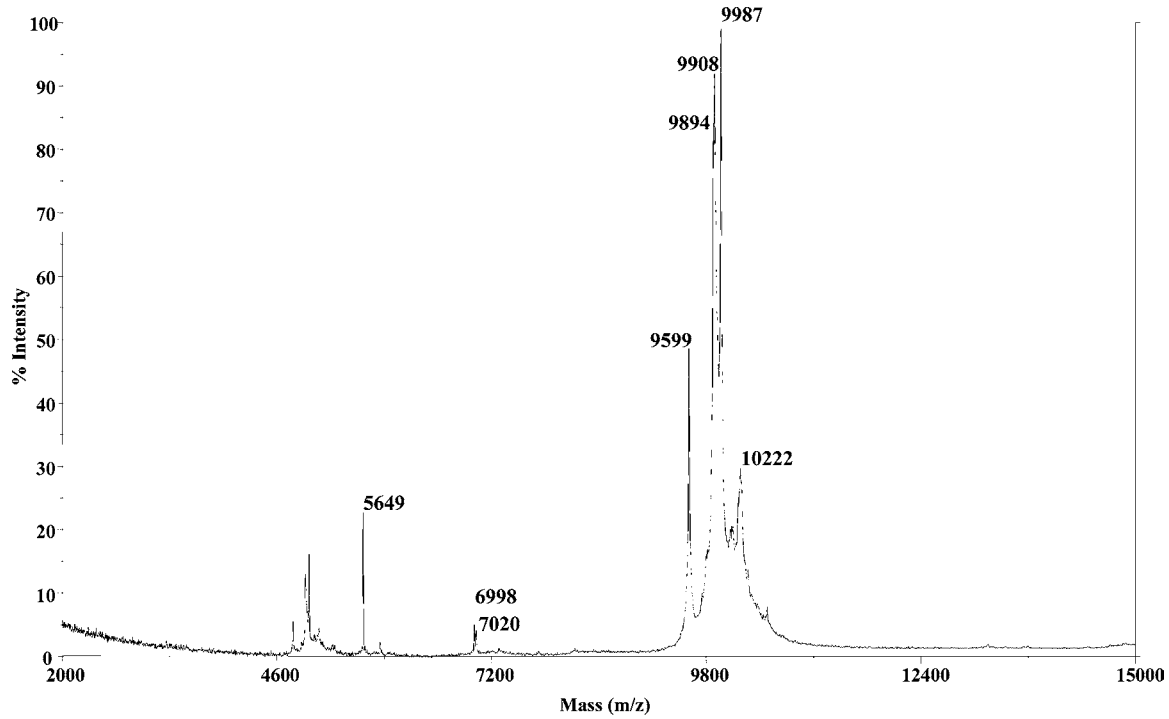

Figure 6. MALDI-TOF mass spectrum of farro pool II G-50 in the *m*/*z* range 2000–15000. The spectrum was obtained using sinapinic acid as matrix and calibrated externally with insulin and cytochrome *c*.

component of 7036 Da (ESI-MS) (**Table 1**) has the same M_r and number of Cys residues as a nsLTP2 from *T. aestivum* (accession no. P82901); a similar protein was also present in *T. durum* seeds (*41*).

The sequences of the first 40 amino acids of the two *T. aestivum* nsLTP2s (P82900 and P82901) indicated that both isoforms differed at amino acid 33, where a glycine is replaced with a proline (*40*). Such a substitution had already been found in nsLTP2 from *V. unguiculata* root hairs (*2*). A second point modification is at residue 58, where threonine is replaced with glutamine.

The 7 kDa protein group of farro pool I G-50 was composed by a more abundant component of 6996 Da (ESI-MS) and by a minor one of 7324 Da (ESI-MS) (**Table 2**). Bioinformatic searches for these two M_r values did not gave any corresponding protein. However, a nsLTP2 with a M_r close to the 7 kDa component has been isolated and characterized from *Oryza sativa* (accession no. P83210, calculated M_r of 7001 Da).

Farro pool II G-50 showed traces of two proteins of the 7 kDa family: a component of 6997 Da and a component of 7019 Da. The first protein has the same M_r of one component already found in farro pool I G-50.

Table 2. Determination by MALDI-MS and RP-HPLC/ESI-MS Analyses of the M_r and Number of Cys Residues of the Protein Components in Farro Pool I G-50 and Results of Bioinformatic Search

unalkylated proteins exptl M_r		alkylated proteins exptl M_r		
MALDI	ESI	MALDI	ESI	bioinformatic search results
7003	6996	7467 (8.14 Cys)	7464 (8.21 Cys)	
7322	7324	7795 (8.29 Cys)	7808 (8.49 Cys)	
9610	9596	10065 (7.99 Cys)	10064 (8.21 Cys)	CAH04989 (*T. aestivum*, calcd M_r 9600.80) S21757 (*T. aestivum*, calcd M_r 9598.82)
9930	9924	10390 (8.07 Cys)	10388 (8.14 Cys)	O04004 (*A. artemisiifolia*, calcd M_r 9921)

Table 3. Determination by MALDI-MS and RP-HPLC/ESI-MS Analyses of the M_r and Number of Cys Residues of the Protein Components in Farro Pool II G-50 and Results of Bioinformatic Search

unalkylated proteins exptl M_r		alkylated proteins exptl M_r		
MALDI	ESI	MALDI	ESI	bioinformatic search results
5648		6112 (8.14 Cys)		
6997		7461 (8.14 Cys)		
7019		7479 (8.14 Cys)		
9598	9596	10063 (8.07 Cys)	10064 (8.21 Cys)	CAH04989 (*T. aestivum*, calcd M_r 9600.80) S21757 (*T. aestivum*, calcd M_r 9598.82)
9893	9892			

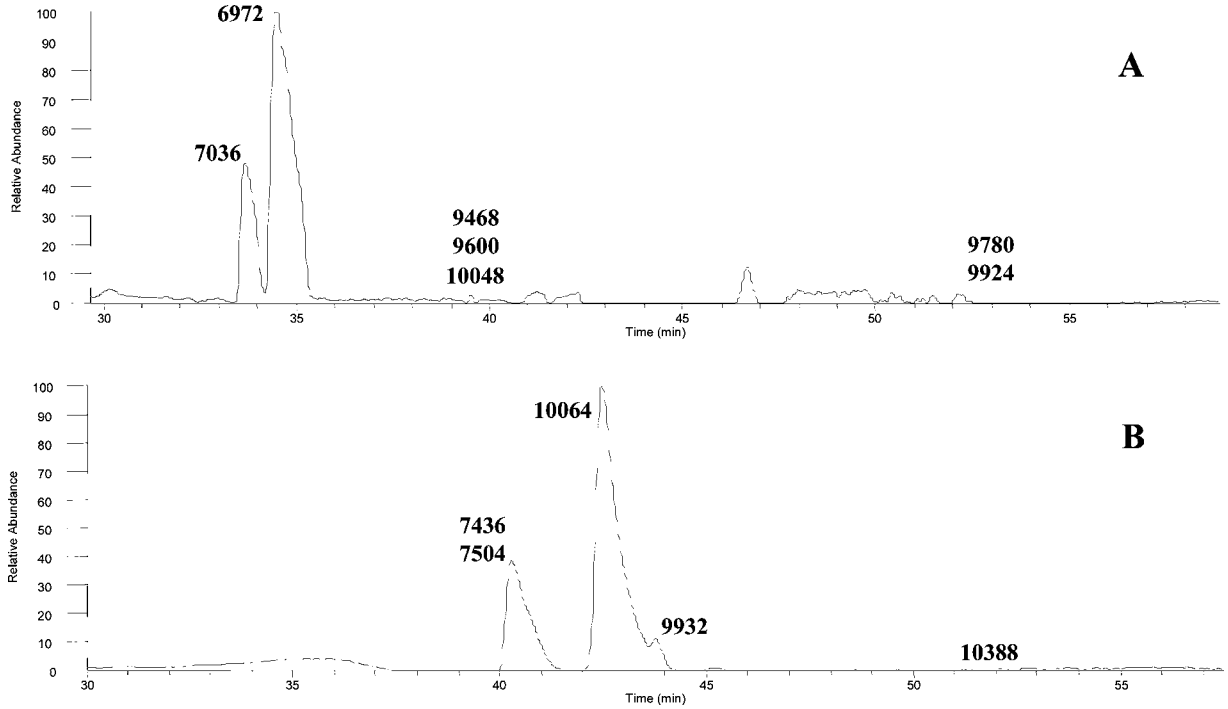

Figure 7. Reconstructed total ion current chromatogram of Centauro pool G-50 (**A**) and reduced and carbamoyl-methylated Centauro pool G-50 (**B**) obtained in the HPLC/ESI-MS analyses.

As with the SDS-PAGE analyses, mass spectra of soft wheat and farro bran extracts showed a second set of proteins corresponding to the nsLTP1 family of 9−10 kDa.

MALDI-TOF and ESI-MS analyses of soft wheat pool G-50 indicated the presence of several proteins with molecular masses of 9468, 9600, 9780, 9924, and 10048 Da (**Table 1**).

Bioinformatic searches for the 9600 Da M_r (ESI-MS) indicated that two proteins isolated from *T. aestivum* (accession no. CAH04989, calculated M_r of 9600.80, and accession no. S21757, calculated M_r of 9598.82) have molecular masses coincident, within the experimental error, with the protein present in the soft wheat bran extract (**Table 1**) (*23*). Although the MALDI-TOF mass spectrum showed a less intense signal for this protein, MALDI-TOF-MS data obtained for the reduced and alkylated mixture and RP-HPLC data demonstrated that this component represents the major one of the soft wheat 9 kDa protein group. The protein purified from *T. aestivum* seeds was found to represent 0.2% of the total seed proteins (*42*). A cDNA clone, encoding a *T. durum* 9 kDa nsLTP (*43*), showed that the deduced primary structure of the mature *T. durum* nsLTP1 was identical to that of the nsLTP1 purified from *T. aestivum*. Subsequently, Douliez et al. (*23*) confirmed the presence of the 9600 Da nsLTP1 in *T. aestivum* and in *T. durum* seeds, together with an isoform (named nsLTP1b) that exhibited a mass excess

of 294 Da, as compared to the native protein. These two isoforms had been already isolated from barley and beer extracts (*19*).

Bioinformatic searches for the other 9 kDa protein components of the soft wheat pool G-50 indicated only one protein isolated from *Ambrosia artemisiifolia* (accession no. O04004, calculated M_r of 9921 Da), showing a molecular mass coincident with the M_r 9924 Da component.

Farro pool I G-50 contained the same 9 kDa proteins already found in soft wheat (9596, 9924, and 10048 Da by ESI-MS). In this case, too, the 9596 Da protein was the major component (**Table 2**).

Today's commercial wheats (durum and bread wheat) are the products of natural hybridization of ancestral types, most of which nowadays are of scarce commercial importance. The high chromosome number of hexaploid wheats contributed to their wider adaptability with respect to species such as the tetraploid and diploid wheats, explaining the great diffusion of these cereal types. By crossing and selecting, man has produced many cultivars of the hexaploid wheat, *T. aestivum*, which today has become the most important wheat species cultivated in Europe, North America, and Australia (*45*). In particular, *T. aestivum* cv. Centauro is the most widespread bread wheat cultivar in northern and central Italy.

On the other hand, the Italian tetraploid farro used in our studies, despite its favorable agronomic traits (reduced fertilization, chemical plant protection, and irrigation), is grown in restricted areas, mainly in central and southern Italy.

As we have demonstrated, the nsLTP1 family in soft wheat and farro bran is constituted basically by the same isoforms, among which the most abundant is the 9599 Da nsLTP1, characteristic of the *Triticum* species. On the contrary, whereas soft wheat showed the nsLTP2 isoforms typical of durum and bread wheats, mass spectral data show that the farro 7 kDa is composed by other proteins. The picture now emerging is that plants are equipped with a large variety of nsLTP genes. These genes probably serve very similar functions but can be specifically turned on in different parts of the plant and in response to a variety of environmental stimuli. The patterns of expression can partially overlap and probably ensure efficient protection and adaptation of the plant to its environment. The duplication events that gave rise to the members of this multigene family probably occurred independently in different species and have led to independent functional specialization. *T. aestivum* cv. Centauro is a species that grows in controlled environments where biotic and abiotic stresses tend to be maximally reduced. In the case of farro, its cultivation survived in marginal mountain areas under harsh ecological conditions on the poorest soils. These different growing conditions could be a possible answer to explain the different patterns of nsLTP2 proteins expressed by these cereals.

Currently, there is considerable interest in the use of ancient wheats, particularly in the health food market. Almost everything and its opposite have been said on farro dietary qualities, whereas almost none of these has been experimentally or analytically verified (*46*). From the scientific side, therefore, skepticism dominates, despite folk knowledge indicating emmer wheats for having positive effects on intestinal regulation and in the treatment of obesity-affected patients. In this perspective, our future work will be focused on the purification of a nsLTP1 from the Centauro cultivar of soft wheat and farro bran and in assaying the stability of these proteins to pepsin hydrolysis as a means to assess their allergenicity.

LITERATURE CITED

(1) Kader, J.-C. Lipid transfer proteins in plants. *Annu. Rev. Physiol. Plant Mol. Biol*. **1996**, *47*, 627−654.

(2) Douliez, J.-P.; Jégou, S.; Pato, C.; Larré, C.; Mollé, D.; Marion, D. Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. *J. Cereal Sci*. **2000**, *32*, 1−20.

(3) Sterk, P.; Booij, H.; Scheleekens, G. A.; Van Kammen, A.; de Vries, S. C. Cell-specific expression of the carrot EP2 lipid transfer protein gene. *Plant Cell* **1991**, *3*, 907−921.

(4) Coutos-Thevénot, P.; Jouenne, T.; Maes, O.; Guerbette, F.; Grosbois, M.; Le Caer, J. P.; Boulay, M.; Deloire, A.; Kader, J. C.; Guerin J. Four 9-kDa proteins excreted by somatic embryos of grapevine are isoforms of lipid transfer proteins. *Eur. J. Biochem*. **1993**, *217*, 885−889.

(5) Torres-Schumann, S.; Godoy, J. A.; Pintor-Toro, J. A. A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. *Plant Mol. Biol*. **1992**, *18*, 749−757.

(6) García-Olmedo, F.; Molina, A.; Segura, A.; Moreno, M. The defensive role of non specific lipid-transfer proteins in plants. *Trends Microbiol*. **1995**, *3*, 72−74.

(7) Treviño, M. B.; O'Connell, M. A. Three drought-responsive members of the nonspecific lipid-transfer protein gene family in *Lycopersicon pennellii* show different developmental patterns of expression. *Plant Physiol.* **1998**, *116*, 1461−1468.

(8) Buhot, N.; Douliez, J.-P.; Jacquemard, A.; Marion, D.; Tran, V.; Maume, B. F.; Milat, M. L.; Ponchet, M.; Mikes, V.; Kader, J.-C.; Blein, J.-P. A lipid transfer protein binds to a receptor involved in the control of plant defense responses. *FEBS Lett.* **2001**, *509*, 27−30.

(9) Blein, J.-P.; Coutos-Thévenot, P.; Marion, D.; Ponchet, M. From elicitins to lipid-transfer proteins: a new insight in cell signalling involved in plant defence mechanisms. *Trends Plant Sci.* **2002**, *7*, 293−296.

(10) Tsuboi, S.; Suga, T.; Takishima, K.; Mamiya, G.; Matsui, K.; Ozeki, Y.; Yamada, M. Organ-specific occurrence and expression of the isoforms of nonspecific lipid transfer protein in castor bean seedlings and molecular cloning of a full-length cDNA for a cotyledon-specific isoform. *J. Biochem*. **1991**, *110*, 823−831.

(11) Koltunow, A. M.; Truettner, J.; Cox, K. H.; Wallroth, M.; Goldberg, R. B. Different temporal and spatial gene expression patterns occur during anther development. *Plant Cell* **1990**, *2*, 1201−1224.

(12) Fleming, A. J.; Mandel, T.; Hofman, S.; Sterk, P.; de Vries, S. C.; Kuhlemeier, C. Expression pattern of a tobacco lipid transfer protein gene within the shoot apex. *Plant J.* **1992**, *2*, 855−862.

(13) Vignols, F.; Wigger, M.; García-Garrido, J. M.; Grellet, F.; Kader, J.-C.; Delseny, M. Rice lipid transfer protein (LTP) genes belong to a complex multigene family and are differentially regulated. *Gene* **1997**, *195*, 177−186.

(14) Jung, H.-W.; Woonbong, K.; Hwang, B. K. Three pathogen-inducible genes encoding lipid transfer protein from pepper are differentially activated by pathogens, abiotic, and environmental stresses. *Plant Cell Environ.* **2003**, *26*, 915−928.

(15) Sossountzov, L.; Ruiz-Avila, L.; Vignols, F.; Jolliot, A.; Arondel, V.; Tchang, F.; Grosbois, M.; Guerbette, F.; Miginiac, E.; Delseny, M.; Puigdomenèch, P.; Kader, J.-C. Spatial and temporal expression of a maize lipid transfer protein gene. *Plant Cell* **1991**, *3*, 923−933.

(16) Tsuboi, S.; Osafune, T.; Tsugeki, R.; Nishimura, M.; Yamada, M. Nonspecific lipid transfer protein in castor bean cotyledons cells: subcellular localization and a possible role in lipid metabolism. *J. Biochem*. **1992**, *111*, 500−508.

(17) Thoma, S.; Kaneko, Y.; Somerville, C. A non-specific lipid transfer protein from *Arabidopsis* is a cell wall protein. *Plant J*. **1993**, *3*, 427−436.

(18) Pyee, J.; Yu, H.; Kolattukudy, P. E. Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. *Arch. Biochem. Biophys*. **1994**, *311*, 460−468.

(19) Jégou, S.; Douliez, J.-P.; Mollé, D.; Boivin, P.; Marion, D. Purification and structural characterization of LTP1 polypeptides from beer. *J. Agric. Food Chem*. **2000**, *48*, 5023−5029.

(20) Jones, B. L.; Marinac, L. A. Purification and partial characterization of a second cysteine proteinase inhibitor from ungerminated barley (*Hordeum vulgare* L.). *J. Agric. Food Chem*. **2000**, *48*, 257−264.

(21) Yu, Y. G.; Chung, C. H.; Fowler, A.; Suh, S. W. Amino acid sequence of a probable amylase/protease inhibitor from rice seeds. *Arch. Biochem. Biophys*. **1988**, *265*, 466−475.

(22) Liu, Y.-J.; Samuel, D.; Lin, C.-H.; Lyu, P.-C. Purification and characterization of a novel 7-kDa non-specific lipid transfer protein-2 from rice (*Oryza sativa*). *Biochem. Biophys. Res. Commun*. **2002**, *294*, 535−540.

(23) Douliez, J.-P.; Jégou, S.; Pato, C.; Larré, C.; Mollé, D.; Marion, D. Identification of a new form of lipid transfer protein (LTP1) in wheat seeds. *J. Agric. Food Chem*. **2001**, *49*, 1805−1808.

(24) Monnét, F.-P.; Dieryck, W.; Boutrot, F.; Joudrier, P.; Gautier, M.-F. Purification, characterization and cDNA cloning of a type 2 (7 kDa) lipid transfer protein from *Triticum durum*. *Plant Sci*. **2001**, *161*, 747−755.

(25) Castro, M. S.; Gerhardt, I. R.; Orrù, S.; Pucci, P.; Bloch, C., Jr. Purification and characterization of a small (7.3 kDa) putative lipid transfer protein from maize seeds. *J. Chromatogr. B* **2003**, *749*, 109−114.

(26) Ramírez-Medeles, M. del C.; Aguilar, M. B.; Miguel, R. N.; Bolaños-García, V. M.; García-Hernández, E.; Soriano-García M. Amino acid sequence, biochemical characterization, and comparative modeling of a nonspecific lipid transfer protein from *Amaranthus hypochondriacus*. *Arch. Biochem. Biophys*. **2003**, *415*, 24−33.

(27) Dubreil, L.; Gaborit, T.; Bouchet, B.; Gallant, D. J.; Broekaert, W. F.; Quillien, L.; Marion, D. Spatial and temporal distribution of the major isoforms of puroindolines (puroindoline-a and puroindoline-b) and non specific lipid transfer protein (ns-LTP1e$_1$) of *Triticum aestivum* seeds. Relationship with their in vitro antifungal properties. *Plant Sci*. **1998**, *138*, 121−135.

(28) Carvalho, A. O.; Machado, O. L. T.; Da Cunha, M.; Santos, I. S.; Gomes, V. M. Antimicrobial peptides and immunolocalization of a LTP in *Vigna unguiculata* seeds. *Plant Physiol. Biochem*. **2001**, *39*, 137−146.

(29) Østergaard, J.; Højrup, P.; Knudsen, J. Amino acid sequences of three acyl-binding/lipid-transfer proteins from rape seedlings. *Biochim. Biophys. Acta* **1995**, *1254*, 169−179.

(30) Sanchez-Monge, R.; Lombardero, M.; Garcia-Selles, F. J.; Barber, D.; Salcedo, D. Lipid-transfer proteins are relevant allergens in fruit allergy. *J. Allergy Clin. Immunol*. **1999**, *103*, 514−519.

(31) Asero, R.; Mistrello, G.; Roncarolo, D.; de Vries, S. C.; Gautier, M. F.; Ciurana, C. L.; Verbeek, E.; Mohammadi, T.; Knul-Brettlova, V.; Akkerdaas, J. H.; Bulder, I.; Aalberse, R. C.; van Ree, R. Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int. Arch. Allergy Immunol*. **2000**, *122*, 20−32.

(32) Ballmer-Weber, B. K. Lipid transfer protein as a potential panallergen. *Allergy* **2002**, *57*, 873−875.

(33) Van Ree, R. Clinical importance of non-specific lipid transfer proteins as food allergens. *Biochem. Soc. Trans.* **2002**, *30*, 910−913.

(34) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(35) Koenig, R.; Stegemann, H.; Francksen, H.; Paul, H. L. Protein subunits in the potato virus X group. Determination of the molecular weights by polyacrylamide electrophoresis. *Biochim. Biophys. Acta* **1970**, *207*, 184−189.

(36) Kussmann, M.; Nordhoff, E.; Rahbek-Nielsen; E.; Haebel, S.; Larsen, M. R.; Jakobsen, L.; Gobom, J.; Mirgorodskaya, E.; Kristensen, A. K.; Palm, L.; Roepstorff, P. Matrix-assisted laser desorption/ionization mass spectrometry sample preparation techniques designed for various peptide and protein analytes. *J. Mass Spectrom.* **1997**, *32*, 593−601.

(37) Gobom, J.; Nordhoff, E.; Mirgorodskaya, E.; Ekman, R.; Roepstorff, P. Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* **1999**, *34*, 105−116.

(38) Castagnaro, A.; García-Olmedo, F. A fatty acid-binding protein from wheat kernels. *FEBS Lett.* **1994**, *349*, 117−119.

(39) De Lamotte F.; Vagner, F.; Pons, J.-L.; Gautier, M.-F.; Roumestand, C.; Delsuc, M.-A. Protein dynamics studies on a wheat type 2 lipid transfer protein. *C. R. Acad. Sci. Paris, Chim.* **2001**, *4*, 839−843.

(40) Douliez, J.-P.; Pato, C.; Rabesona, H.; Mollé, D.; Marion, D. Disulfide bond assignment, lipid transfer activity and secondary structure of a 7-kDa plant lipid transfer protein, LTP2. *Eur. J. Biochem*. **2001**, *268*, 1400−1403.

(41) Monnét, F.-P. Caractérization d'une protéine de fixation de lipides du blé dur, purification, sequençage, ADN complémentaire, relations aux protéines végétales de transfert de lipides et aux inhibiteurs d'α-amylase/trypsine des céréales. Ph.D. Thesis, USTL, Montpellier, 1990.

(42) Désormeaux, A.; Blochet, J.-E.; Pézolet, M.; Marion, D. Amino acid sequence of a non-specific wheat phospholipid transfer protein and its conformation as revealed by infrared and Raman spectroscopy. Role of disulfide bridges and phospholipids in the stabilization of the α-helix structure. *Biochim. Biophys. Acta* **1992**, *1121*, 137−152.

(43) Dieryck, W.; Gautier, M.-F.; Lullien, V.; Joudrier, P. Nucleotide sequence of a cDNA encoding a lipid transfer protein from wheat (*Triticum durum* Desf.). *Plant Mol. Biol*. **1992**, *19*, 707−709.

(44) Arondel, V.; Vergnolle, C.; Cantrel, C.; Kader, J.-C. Lipid transfer proteins are encoded by a small multigene family in *Arabidopsis thaliana*. *Plant Sci*. **2000**, *157*, 1−12.

(45) Belderock, B. Developments in bread-making processes. *Plant Food Hum. Nutr*. **2000**, *55*, 1−14.

(46) D'Antuono, L. F.; Bravi, R. The hulled wheats industry: present developments and impact on genetic resources conservation. In *Hulled Wheat. Proceedings of the First International Workshop on Hulled Wheats*, July 21−22, 1995, Castelvecchio Pascoli, Tuscany, Italy; Padulosi, S., Hemmer, K., Heller, T., Eds.; IPGRI: Rome, 1995; pp 221−233.

Received for review February 11, 2005. Revised manuscript received July 14, 2005. Accepted July 25, 2005. This work was partially funded by MIUR as part ot the 2002 PRIN project "Biochemical, genetical and molecular aspects of wheat kernel proteins in relationship to the nutritional and technological characteristics of derived end products".

JF0580465