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Proteome from Lemon Fruit Flavedo Reveals That This Tissue Produces High Amounts of the Cit s1 Germin-like Isoforms

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A multistep procedure has been developed and applied to extract and purify proteins from lemon fruit flavedo. 2DE, LC-ESI-MS/MS, and bioinformatics were used to detect the high abundance of the germin-like glycoprotein Cit s1, a powerful allergen in humans. Peptide alignments against *Citrus* EST repositories gave the best scores with the *C. sinensis* cDNA (gi|188354270/EY710037), annotated as unknown *sweet orange fruit protein*; additional BLAST of peptides against NCBI databases gave high sequence identities with sequence of orange Cit s1 (gi|52782810/P84159), suggesting that the unknown *sweet orange fruit protein* is consistent with the Cit s1 protein. Peptides of Cit s1 were detected in 17 spots ranging from 120 to 20 kDa, pointing out that in the flavedo of lemon the Cit s1 may be expressed as several isoforms of which the 120 kDa isoform is the largest monomer and the 20 kDa is the smallest one. This finding adds information about the features of Cit s1, because it has been previously reported as a unique monomeric glycoprotein of 24 kDa.

KEYWORDS: Lemon flavedo; proteome; Cit s1; germin-like protein; plant allergens

INTRODUCTION

Lemon is a traditional crop of the Mediterranean area. Morphologically, the mature lemon fruit is composed of the epicarp, known also as the flavedo, the mesocarp, known as the albedo, and the endocarp, formed by large juice sacs that accumulate sugars and organic acid. The flavedo is formed by a mosaic of epidermal cells and glands, syncytia, the cells of which produce high amounts of essential oil, pouring them in the large gland lumen (*1*). Lemon fruit juice is used to make refreshing drinks, whereas essential oils are extracted from lemon peel and used in scent or in foodstuff preparations as additives.

The benefits of citrus fruit products on human health are mainly due to their high content of antioxidant molecules (*1*). Despite the positive effects, many people suffer allergy syndromes against molecules such as terpenes, glycans, and glycoproteins produced and accumulated in the fruits of species of the *Citrus* genus. Among the citrus allergens, the orange protein Cit s1 causes a strong elicitation of IgE in patients as evidenced in extensive clinical studies (*2*). Cit s1 constitutes one of three recently identified and characterized orange allergens, of which Cit s2 corresponds to orange profilin (*3*) and Cit s3 belongs to the lipid transfer proteins (*4*). Cit s1, on the other hand, was first detected in a study of six patients as being a protein of 24 kDa recognized by patients' IgE (*5*), which in a larger study was shown to display high *in vitro* reactivity, with its glycans constituting the major IgE epitopes (*2*). Cit s1 was also identified in an independent study as being a major orange allergen (*6*). Initial N-terminal sequencing data indicated that Cit s1 may be a germin-like protein (*2*, *6*), whereas the full sequence of Cit s1 was, more recently, determined, and the nature of its N-glycosylation has been examined using MS, in terms of its peptide and glycan sequence (*7*). Even in these cases the patients' sera IgE recognized the 24 kDa Cit s1 glycoprotein as a unique antigen. The biological activity of Cit s1 in flavedo tissue remains, up to now, to be clarified. Germin and germin-like proteins are cell wall glycoproteins with robust quaternary structures that show an unusual resistance to detergent treatment, heat denaturation, and degradation by proteases (*8*). They constitute a large and varied family of plant proteins, belonging the cupin superfamily, participating in many processes that are important for plant development and defense, showing a number of functions, from antioxidative metabolism (oxalate oxidase, superoxide dismutase, etc.) to signal reception and transduction or in cell wall strengthening and resistance to pathogen attack and abiotic stress (*9*−*11*). They can also act as peroxidase (*8*) or superoxide dismutase, activities in extracellular compartments (*12*). The germin-like proteins have typically a hexameric quaternary structure resulting from the assemblage of trimers of dimers, although there are examples of different oligomerization (*13*). Each monomer's sequence showed a conserved motif derivative from that of the cupin superfamily (*9*, *13*). Glycosylation may be responsible for the shift between the predicted size of the monomer and the molecular weight from the electrophoresis separation. In the case of Cit s1 a single *N*-glycan is the target of the IgE response (*2*, *7*).

Proteomics has become a powerful tool in plant research in the past few years. The development of the LC-ESI-MS/MS technology, excellent separation techniques, development of genomic, and EST databases for a variety of species and powerful bioinformatics tools enable the understanding and assessment of protein

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function, their relative abundance, the modifications affecting enzyme activity, their interaction with other proteins, and subcellular localization. In the past decade proteomics research has been conducted in several plant species mainly using 2DE gels. Most successful studies are those which use separation of subcellular compartments such as mitochondria (*14*), chloroplast (*15*), endoplasmic reticulum (*16*), peroxisomes (*17*), and plastoglobules (*18*), because they contain a limited number of proteins that help in protein identification. For the *Citrus* genus, proteome analysis was improved by the large-scale sequencing project and the availability of the EST database that is a fundamental part of genomics research to enable gene discovery and annotation. The citrus genome-wide EST database, including prevalently information from the juice sac tissue, consists of 157,608 clones corresponding to 19,854 contigs and 37,138 singletons (http://cgf.ucdavis.edu). From that, even more protein sequences from orange, mandarin, and related species have been recognized and annotated in the databases. The function assigned to proteins and the elucidation of the related metabolism in which the proteins act are escalating, especially for key processes involved in the development and maturation of fruit (*19*−*21*).

We decided to analyze the proteome of lemon flavedo, taking advantage of the *Citrus* database, with the aim to obtain the genomic characterization at protein level of this tissue undergoing environmental stress, pathogen attack, and treatment with pesticides employed in crop management. Here, we describe the first attempt to analyze lemon proteome using two-dimensional electrophoresis and LC-ESI-MS/MS, focusing on mature flavedo cells and aiming at the identification of proteins specifically expressed.

MATERIAL AND METHODS

Plant Material. In Rocca Imperiale, a nice locality in Calabria (southern Italy), a selected lemon crop (var. Femminello) grown under optimal conditions of climate and in organic cultivations produces high-quality fruits. In particular, the fruit's flavedo is characterized by a larger number of glands containing a high amount of limonene (up to70%) with respect to other genotypes of the same grove (*22*). Lemon fruits ($n = 5$) showing a full yellow flavedo were harvested in December at 260 days after flowering (DAF), from five trees, for a total amount of 25 fruits. By means of a blade, sections of flavedo tissue were excised from each harvested fruit. Tissue samples were pooled, immediately frozen in liquid N_2, and divided into aliquots of about 1 g each, which were the independent biological replicates for protein extraction.

Protein Extraction and Purification. We tested different protein extraction methods (*23*−*25*) to obtain the flavedo protein extracts suitable for 2DE. One aliquot of flavedo tissue (1 g) was ground in liquid N_2 to obtain a fine powder by the aid of quartz sand (silicon dioxide) and then transferred in 2 mL microtubes. Tissue powder was processed separately according to three different procedures as follows: (a) The first procedure involved dissolving the tissue powder with 100% cold acetone (AC procedure); the sample was vortexed for 30 s and centrifuged at 13000 rpm for 3 min (4 °C). The obtained pellet was extensively washed with cold 10% TCA in acetone until the supernatant became colorless and once with cold aqueous 10% TCA. Then the pellet was rinsed twice with cold 80% acetone, using both vortex and centrifugation in every step. The final tissue powder was dried at room temperature and used for protein extraction or stored at −80 °C for further use. (b) In the second procedure cold 10% TCA in acetone (TCA−AC procedure) was added to the tissue powder and washed three times; afterward, the pellet was extensively washed in 10% TCA. The TCA was removed by washes with cold acetone until the supernatant was colorless and was dried at room temperature. (c) In the last procedure, 20% aqueous TCA (TCA procedure) was added to the tissue powder. After the vortexing, the resultant pellet was washed once more with 20% TCA. The pellet was rinsed with cold 80% acetone, vortexed, and centrifuged until the supernatant was colorless. The final pellet was dried at room temperature.

The protein purification was made from AC, TCA−AC, and TCA tissue powders, following the phenol extraction methods optimized by Wang et al. (*25*) for recalcitrant plants. About 0.1 g of the dry tissue powder was dissolved in 0.8 mL of phenol (Tris-buffered, pH 8.0; Sigma, St. Louis, MO) and 0.8 mL of dense SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl buffer, pH 8.0, 5% 2-mercaptoetanol) in a 2 mL microtube. Samples were vortexed for 30 s and centrifuged at 13000 rpm for 5 min. The upper phenol phase was removed and pipetted to fresh microtubes (0.3 mL for 2 mL tube), 5 volumes of cold methanol plus 0.1 M ammonium acetate was added, and the mixture was stored at −20 °C for 30 min. The precipitated proteins were recovered by centrifugation at 13000 rpm for 5 min and then washed with cold methanolic ammonium acetate twice and cold 80% acetone twice. The final pellet was dried and dissolved in Laemmli sample buffer for 1DE separation or in 2DE rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; 50 mg/mL DTT).

Electrophoresis and 2DE Gel Analysis. For 2DE analyses, rehydrated protein samples were loaded on a 7 cm Immobiline DryStrip (pH 3−10; Bio-Rad) overnight. First-dimension isoelectrofocusing (IEF) was performed in an IPGphor system (Bio-Rad). Focused strips were equilibrated using 2% DTT and 2.5% iodoacetamide solutions and then positioned on a 12.5% gel. Denaturant second dimension (SDS-PAGE) was carried out under a constant current of 45 mA/gel. The obtained protein maps were stained with CBB and scanned on the densitometer GS-800 (Bio-Rad), and images were stored by PDQuest Basic 2-D analysis software (Bio-Rad). Automated spot detection and matching make pairwise comparisons between spots in 10 gels from five biological replicates, allowing the separation of merged and overlapping spots (PDQuest Advanced, Bio-Rad), thus obtaining a master gel image containing all of the spots that are in at least five gels. In the master gel the peptides are mapped with reference to molecular weights (MW) and isoelectric points (p*I*). For each spot, area and volume were calculated. After background subtraction, spot volume was normalized as the percentage of the total volume of protein spots on the same gel, so that the modal peak of volume ratios was zero. From this analysis it is possible to evaluate the relative abundance of all spots in protein samples.

LC-MS/MS and Protein Identification. Gel plugs containing protein spots of interest were manually excised and digested overnight with trypsin at 37 °C (*26*). The tryptic fragments were analyzed by LC-ESI-MS/MS. For the experiments, a Bruker HTC Ultra spectrometer, equipped with a Dionex Ultimate 3000 HPLC system, was used. Chromatography separations were conducted on a Thermo Biobasic C18 column (1 mm i.d. × 100 mm length and 5 μm particle size), using a linear gradient from 5 to 90% acetonitrile (ACN), containing 0.1% formic acid with a flow of 100 μL/min, including the regeneration step; one run lasted 70 min. Acquisitions were performed in the data-dependent MS/MS scanning mode (full MS scan range of m/z 250−2000 followed by full MS/MS scan for the most intense ion from the MS scan).

Protein identity was searched after peptide sequence attributions by using Global Proteome Machine (GPM) software (www.thegpm.org) against available plant databases (including *Citrus sinensis*).

Peptide sequences that were not identified through this database search method were further analyzed for a de novo peptide sequencing with the PepNovo Sequencing Algorithm (*27*), by means of which the determination of each original peptide sequence is done without knowledge of the genomic sequences as well as the help of a protein database. Finally, all peptides were interrogated using MS-BLAST software (Basic Local Alignment Tool Mass Spectrometry, http://dove.embl-heidelberg.de/Blast2/msblast.html), in order to get the putative protein identity. In some spots, more than one protein was identified.

RESULTS

Purification of Proteins and 2DE Electrophoresis. As expected, lemon flavedo was revealed as a tissue recalcitrant to common protein extraction methods. In fact, when we tried to extract proteins by direct homogenization of tissue in aqueous phosphate buffers, the obtained protein pellets were hard to dissolve in electrophoresis buffers and caused vertical and horizontal streaking and high background even in the first electrophoresis dimension gels. This is due to the occurrence of high contamination of

pectins, sugars, monoterpenes, and other contaminants that copurify with the proteins. Thus, the removal of pectin and other compounds, prior of the protein purification, has been necessarily required. The efficiency of contaminant removal of the three different procedures was evaluated by the quality of protein extracts obtained from flavedo and analyzed by 1DE patterns (**Figure 1**). Better results were obtained with the TCA−AC procedure (**Figure 1c**), which gave a high number of protein bands well resolved, especially at low molecular weights that were poorly detected in protein patterns from both the AC and TCA procedures (**Figure 1a**,**b**). The proteins extracted by the TCA−AC procedure were subjected to 2DE gel electrophoresis, and peptide spots were detected by CBB staining. In each gel can be distinguished no more than 180 spots; however, automated spot detection and matching gave more than 400 different spots overall mapped in 10 gels, revealing a low overlapping of the spots among replicates. Low overlapping of spots is essentially due to changes in experimental conditions among the independent extractions and separations of proteins. Consequently, pairwise comparisons of spots in the gels of all replicates gave a map of ca. 120 spots in the master gel (**Figure 2**), whereas about 60 spots vary their molecular weight and/or isoelectric point in each gel. On the basis of NV values of all peptide spots it could be possible to evaluate their relative abundance in protein extracts that it may be linked both to efficiency in protein extraction from tissue and also to level of protein expression in cells; spots for which NV values are between 7 to 3 have been considered to be *highly* abundant, spots from having NV values from 2 to 1 are *intermediately* abundant (middle), and spots having values of < 1 have been considered as *poorly* or *very poorly* abundant spots (low or very low). Among the 120 spots in the master gel, one of them has an NV value of 15 (**Figure 2**, spot 40), whereas the percentage of remaining spots in each interval was 13% of spots with NV values up to 3.5, 22% of spots with NV values up to 1.5, 36% of spots with NV values up to 0.5, and a large number of spots (28%) having NV values of < 0.5.

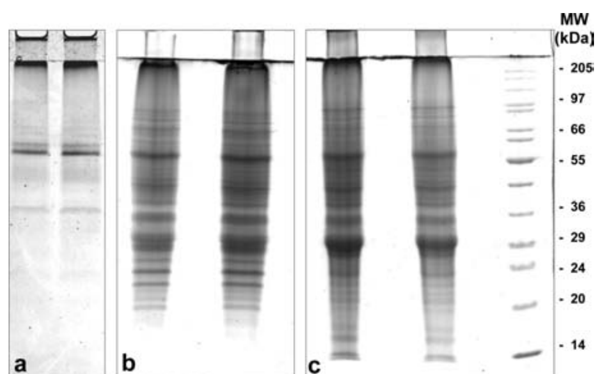

Figure 1. CBB-stained 1DE gels of protein extracted from lemon flavedo by means of (**a**) the AC procedure, (**b**) the TCA procedure, and (**c**) the TCA−AC procedure. See the text for details.

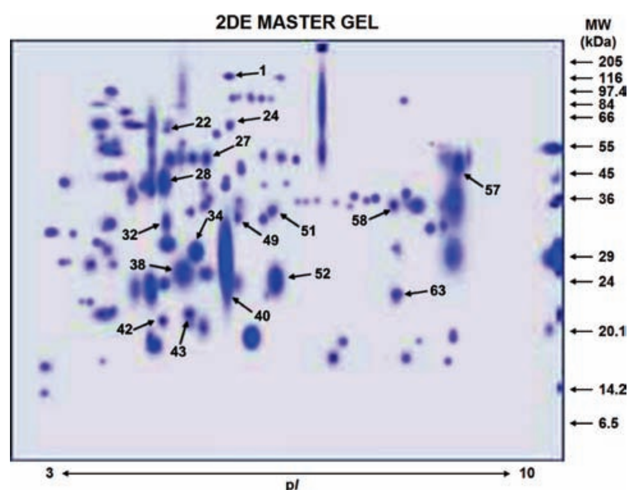

Figure 2. CCB-stained 2DE master gel of proteins extracted from lemon flavedo. The image of the master gel includes all of the spots that are at least in 10 independent gels from 5 biological replicates. Spots are mapped with reference to their molecular weights (MW) and isoelectric points (p*I*). Arrows indicate the spots where the peptide fragments of the Cit s1 have been detected.

Protein Identification by Mass Spectroscopy and Bioinformatics. We have restricted the sequence analysis to only spots detected in all replicates; even if this approach has eliminated any inaccuracies, it certainly has excluded potentially interesting proteins, which can be further considered in a global proteome analysis of flavedo.

Among the spots highly and intermediately abundant we were able to identify 60 proteins; the remaining spots were either too close or too weak to be isolated. Nevertheless, spots were excised

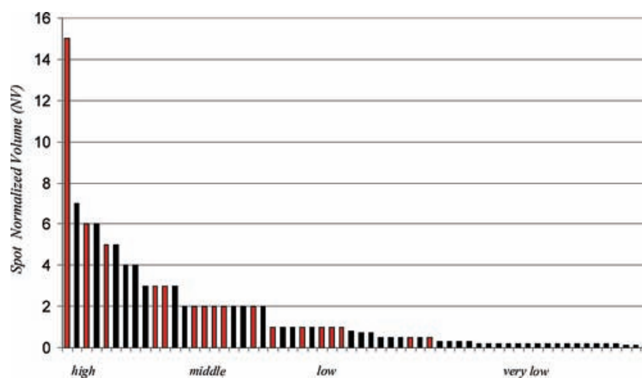

Figure 3. Range of normalized volume (NV) of spots including those where the peptide fragments of the Cit s1 have been detected (red bars). Higher NV values correspond to higher abundant proteins in each spot, whereas lower NV values correspond to lower abundant proteins.

Table 1. Percentage of Identified Proteins in Lemon Flavedo, Grouped by Their Assigned Function and Related Metabolism

identified proteins (%)	assigned function	metabolism
23	heat shock, agglutination, pathogen-related proteins	stress and defense
11	oxidative phosphorylation, enzymes in the citric acid cycle, glycolysis	mitochondrial metabolism
9	carbon uptake, gluconeogenesis, glycolysis	sugar anabolism, chloroplast metabolism
11	antioxidant, peroxidation, redox buffering	detoxification and antioxidant metabolism
26	"sweet orange" proteins of *Citrus* genus, no assigned function	ND[a]
18	not classified	ND
2	Cit s1 glycoprotein of still unknown function, human allergen	ND

[a] ND, not determined.

Table 2. Peptide Sequences, Sequence Identities, and Protein Attribution of Tryptic Fragments Detected in the 17 Spots in All Replicate 2DE Gels[a]

spot	MW	p*I*	log(e) protein	attribution/residues alignment	detected peptide sequence	log(e) peptide
42	20.0	5.0	−5.4	gil188354270/aa 89−113	LGVDETDANVEQIPGLNTLGISAFR	−5.4
28	43.5	5.1	−15.3	gil188354270/aa 37−53; 89−113	TDPGHLQDVC VAINDPK	−3.9
					LGVDETDANVEQIPGLNTLGISAFR	−7.4
32	33.5	5.1	−20.5	gil188354270/aa 89−113; 71−88	LGVDETDANVEQIPGLNTLGISAFR	−7.7
					AEDFFFSGLGKPGNTANR	−8.9
22	66	5.2	−3.9	gil1883542707 aa 89−113	LGVDETDANVEQIPGLNTLGISAFR	−3.9
43	22	5.4	−5.0	gil188354270/aa 89−113	LGVDETDANVEQIPGLNTLGISAFR	−5.0
34	29.5	5.5	−4.3	gil188354270/aa 89−113	LGVDETDANVEQIPGLNTLGISAFR	−4.3
27	53.5	5.8	−8.9	gil188354270/aa 89−113	LGVDETDANVEQIPGLNTLGISAFR	−8.9
1	120	6.0	−22.2	gil188354270/aa 71−88; 89−113	AEDFFFSGLGKPGNTANR	−8.8
					LGVDETDANVEQIPGLNTLGISAFR	−9.9
38	26	5.3	−55.0	gil188354270/aa 37−53; 71−88; 71−84; 71−81; 89−113	TDPGHLQDVC VAINDPK	−6.6
					AEDFFFSGLG KPGNTANR	−9.8
					AEDFFFSGLG KPGN	−2.3
					AEDFFFSGLG K	−2.4
					LGVDETDANV EQIPGLNTLG ISAFR	−7.6
40	28	6.0	−77.6	gil188354270/aa 35−53; 37−53; 71−88; 89−113; 89−110; 89−107; 93−113	SATDPGHLQDVCVAINDPK	−4.8
					TDPGHLQDVCVAINDPK	−5.4
					AEDFFFSGLGKPGNTANR	−8.8
					LGVDETDANV EQIPGLNTLG ISAFR	−8.4
					LGVDETDANVEQIPGLNTLG IS	−2.0
					LGVDETDANVEQIPGLNTL	−2.2
24	65	6.0	−7.6	gil188354270/aa 89−113	LGVDETDANVEQIPGLNTLGISAFR	−7.6
49	34	6.2	−7.3	gil188354270/aa 89−113	LGVDETDANVEQIPGLNTLGISAFR	−7.3
51	35	6.8	−7.5	gil188354270/aa 89−113	LGVDETDANVEQIPGLNTLGISAFR	−7.5
52	25	6.8	−30.6	gil188354270/71−88; 71−82; 89−113	AEDFFFSGLG KPGNTANR	−8.3
					AEDFFFSGLGK	−2.4
					LGVDETDANV EQIPGLNTLG ISAFR	−8.7
58	36	8.5	−5.7	gil188354270/aa 89−113	LGVDETDANVEQIPGLNTLGISAFR	−5.7
63	23	8.6	−7.7	gil188354270/aa 89−113	LGVDETDANVEQIPGLNTLGISAFR	−7.7
57	50	9.5	−6.0	gil1883542707 aa 89−113	LGVDETDANVEQIPGLNTLGISAFR	−6.0

[a] Spots are reported according with their molecular weight (MW) and isoelectric point (p*I*).

from the gels, and protein in-gel digestion was performed. Using LC-ESI-MS/MS analyses and GPM software search, 60 proteins were unambiguously identified. We found correspondence with identified proteins and enzymes involved in various biosynthetic pathways. In particular, many proteins are involved in primary metabolisms, such as photophosphorylation and oxidative phosphorylation, whereas others belong to oxidative stress and secondary metabolisms (**Table 1**).

The most highly abundant spot corresponds to enzymes involved in the detoxification and antioxidant metabolism. Some proteins were classified as "sweet orange proteins", the functions of which are still unknown.

As a main result we found that the trypsin-digested fragments from 17 spots gave some identical peptide sequences (**Figure 2**, see arrows). In **Figure 3** the distribution of NV values of spots in which identical peptides were found and their related abundance intervals have been reported; these spots (red bars) are among those with high and intermediate (middle) abundance NV values. The sequences of peptides and spots in which each was detected are reported in **Table 2**; as can be seen, the peptides were found in a spot with highest molecular weight of 120 kDa, as in a lowest one with 20 kDa molecular weight. Peptides were detected also in spots with intermediate molecular masses (66, 65, 55, 42, 36 kDa). Their isoelectric points also vary from anionic (5.0 pH units) to cationic (9.5 pH units). LC-MS/MS of each peptide followed by GPM against the *Citrus* EST repositories gave high score matching with the EST CS00-C3-701-111-B12-CT.F (gi|188354270), an unknown protein annotated as *Sweet orange fruit protein, development stadium* (*2 of 6*) *Citrus sinensis*. Peptides, except one, gave full alignment at the 35−53 and 71−103 aa residues of the deduced protein sequence (**Figure 4a**; **Table 2**).

BLAST search of all peptides against the common databases gave the best sequence alignment of the peptide SATDPGHLQDVCVAINDPK at the 3−19 residues of the N-terminal sequence

a

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*LKFEIGTSCDLITMKVIQFLIGFALLALASSLASATDPGHLQDVCVAINDPKDGVFVNG          60
KFCKDPKLARAEDFFFSGLGKPGNTANRLGVDETDANVEQIPGLNTLGISAFRIDYAPKW        120
PKATSHSPPCQQKIFFIPEKENLYVRYLNFTPNE*RPFLLKNSK*RKKFFRSNHRERNSS        180
SHFPLRKRTKCQRHITGS*HKEIPKGENI*TPGR*RLEDKTEIRHKD*IRKRKE*KTDGE        240
RKRKGGIKKKKGVEKRQKDKIDGKKKNTKEETEEEEKQQKQKKHTDSKETSHKEDENNVK        300
LTNITRTIRVENKYTMTLGIKYIKIGKWHYAKNAK*PHELETGTMMS*NDTE*CETGMGT        360
AGRDDRQTWQRTEAGRRADAK                                               381
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b

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MKVIQFLIGFALLALASSLASATDPGHLQDFCVAINDPKDGVFVNGKFCKDP                 52
KLAR[AEDFFFSGLGKPGNTANR] [LGVDETDANVEQIPGLNTLGISAFR] IDYA             103
PYGQRPPHIPRASEIFLVLGTLYVGFVTSDQLN NTLIAKALNKGDVFVFPQG                155
LIHFQFNIGK [TNAAAYSALNSQFPGEVTIADTVFGANPSINPDFLGK] AFQLD              207
PKIVKDLQNKFINGN
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Figure 4. Translated sequences of (**a**) *Citrus sinensis* cDNA gi| 188354270 and (**b**) putative Cit s1 open-reading frame. The highlighted peptides are those detected in our study. The sequences in the boxes have also been confirmed by MS analysis in an independent study (*7*).

for the germin-like protein Cit s 1 from *C. sinensis*, annotated in the UniProtKB database source with the accession no. P84159. The peptide is the same that aligns at the 35−53 aa residues of the gi|188354270 EST sequence (**Figure 4a**).

In a parallel analysis to GPM and BLAST searches, we found full alignment of all peptides at the deduced open-reading frame sequence for Cit s1, published by Pöltl et al. (*7*), but never annotated in databases; in particular, the largest peptide, TNAAAYSALNSQFPGEVTIADTVFGANPSINPDFLGK, found in eight spots (34, 38, 40, 49, 52, 57, 58, 63) has full sequence coverage at 168−204 aa residues. On the other hand, the same peptide had not already produced significant alignments against the *Citrus* EST repositories.

DISCUSSION

Proteomics of lemon flavedo pointed out that this tissue is able to synthesize large amounts of Cit s1, as can be deduced by high density values of the spots in which its peptides were found. This glycoprotein was previously identified as a new class of major allergen in orange fruit allergy, and it was recently denominated in the *International Union of Immunological Societies* (IUIS) allergen database; despite this, little information on its assigned function in planta and clinical relevance has been published so far (*4*, *6*, *7*). It is known that the allergen shows persistent IgE-binding capacity during industrial preparation of orange juice, which included pasteurization, and may thus contribute to allergic reaction after ingestion of orange fruits and heat-processed juice (*6*). Several independent studies reported that orange Cit s1 is a 24−25 kDa glycoprotein that was partially purified and subsequently identified as a germin-like protein by N-terminal amino acid sequencing, molecular size, and recognition by rabbit anticomplex N-linked glycan antibodies (*2*, *5*).

As a main result, we were able to recognize that the mRNA sequence (gi|188354270) from orange fruit, annotated in the *Citrus* EST repositories as the unknown *Sweet orange fruit protein, development stadium* (*2 of 6*), has identity with the N-terminal sequence of germin-like Cit s1 glycoprotein (P84159, UniProtKB source). The identity of two sequences has not been reported yet, but our finding will allow us to annotate the new protein record for Cit s1 in *Citrus* EST databases.

As well, peptide fragments revealed by LC-MS/MS and GPM software are also coincident with the largest ones obtained by Pöltl et al. (*7*) after tryptic digestion of the partially purified Cit s1 from orange peel extract. On the bases of all peptide matching among the identified sequences we found, no certain attribution to the Cit s1 protein of the largest fragment TNAAAYSALNSQFPGEVTIADTVFGANPSINPDFLGK can be assigned; further purifications of the protein and its complete sequencing, especially at the C-terminal sequence, are therefore needed.

The structure of the native lemon Cit s1 is still unknown; for germin-like proteins, examples of different oligomerizations have been reported in plant species, even the typically homohexameric quaternary structure resulting from the assemblage of trimers of dimers (*13*). Our findings point out that Cit s1 peptides were detected in 17 spots with the highest molecular mass of 120 kDa and the lowest of 20 kDa, which may correspond to many Cit s1 isoforms having monomers differing notably in size. This finding could be expected because germin-like proteins belong to the cupin superfamily, which includes several gene members (*9*−*11*). Otherwise, we can assume an anomalous migration in 2DE as those reported previously by germins and germin-like proteins on SDS-PAGE (*28*). In both cotton fiber and barley leaf samples, native oligomeric germin-like proteins migrated with an apparent molecular mass of 108 kDa (*29*). After heating and in the presence of dithiothreitol, germin-like proteins migrated as a prominent band at approximately 50−52 kDa and fainter bands at 25 and 27 kDa. It is likely that the 50−52 kDa band corresponds to germin-like protein dimers, a form of intermediate stability under denaturing conditions in both cotton fiber and barley leaf samples (*28*, *30*). On the basis of similarity with the wheat and barley germin features, we can suppose that the range of molecular weights between 66 and 40 kDa we found could be consistent with the rearrangement of the 20−25 kDa monomers in such oligomer (trimers and dimers) intermediate forms, even if our results do not provide suitable evidence. On the other hand, the peptide of 120 kDa should be a monomer, because it is very unlikely that an oligomeric protein, resulting from an arrangement of at least six 20 kDa monomers, can migrate under denaturing conditions. Any way our results shall be considered, there is no doubt that they add information about the features of Cit s1 reported by several independent studies on *Citrus*, in which protein was a monomeric 24 kDa glycoprotein (*2*, *6*, *7*).

As a last result to be discussed, whole proteomic analyses of lemon flavedo showed that detoxification and antioxidant are two major metabolisms in mature tissue. The occurrence of enzymes belonging to the glycolytic and gluconeogenesis pathways is noteworthy because it suggests the capacity of the flavedo cells for energy production and carbon assimilation. Proteins related to defense response and stress are also highly expressed, indicating that flavedo is a powerful barrier against biotic and abiotic aggressions. We were not able to identify further peptides in the remaining 10 spots, even when their sequence identities were checked in the *Citrus* EST databases in which the greater part of deposed sequences are from endocarp juice sacs. This could mean that the unidentified proteins have not been isolated and sequenced yet; on the other hand, some of them could be selectively expressed in lemon flavedo and/or they absolved their roles in their own metabolic pathways, emphasizing the importance of further proteomic studies.

ABBREVIATIONS USED

2DE, two-dimensional electrophoresis; PAGE, polyacrylamide gel electrophoresis; LC-ESI-MS/MS, liquid chromatography−electrospray ionization−tandem mass spectrometry; MS, mass spectrometry; ESTs, expressed sequence tags; SDS, sodium dodecyl sulfate; DTT, 1,4-dithiothreitol; TCA, trichloroacetic

acid; CBB, Coomassie Brilliant Blue R-250; GPM, Global Proteome Machine; MS-BLAST, Basic Local Alignment Tool Mass Spectrometry.

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