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Comprehensive proteome analysis of lettuce latex using multidimensional protein-identification technology

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ABSTRACT

Commercially, lettuce (*Lactuca sativa*) is one of the most important leafy vegetables. Lettuce produces a milky latex of variable chemical compositions within its laticifers. As a step toward understanding the main physiological roles of this latex in higher plants, we embarked on its proteomic analysis. We investigated 587 latex proteins that were identified from the lettuce latex using multidimensional protein-identification technology. A bioinformatics analysis showed that the most frequently encountered proteins in the latex were organellar proteins from plastids and mitochondria, followed by nucleic and cytoplasmic proteins. Functional classification of the identified proteins showed that proteins related to metabolism, cell rescue, defense, and virulence were the most abundant in lettuce latex. Furthermore, numerous resistance proteins of lettuce and viral proteins were present in the latex suggesting for the first time a possible function of the lettuce latex in defense or pathogenesis. To the knowledge of the authors, this is the first large-scale proteome analysis of lettuce latex.

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1. Introduction

Commercially, lettuce (*Lactuca sativa*) is one of the most important leafy vegetables. It is the most popular salad vegetable in the world, which is consumed mostly as a fresh product. Lettuce belongs to the family *Asteraceae* and contains nine pairs of chromosomes. Lettuce secretes a colored juice that is often milky called latex. The latex accumulates in specialized continuous tubes called laticifers. Laticifers are specialized cells that can be found in more than 20 plant families including the *Asteraceae* family, the poppy family, Greater Celandine, and rubber trees, among others (Pickard, 2008). Laticifers seem to have polyphyletic origins. Therefore, their classification is based on morphological characteristics and developmental patterns. The primary role of laticifers is to provide a place to deposit numerous metabolites which might be synthesized from several cellular compartments, such as plastids and mitochondria. Although all latex-containing plants produce such kinds of metabolites, increasing studies have demonstrated that the latex in the laticifer is important for defense mechanism against insects. Furthermore, in a recent review paper it was described very well that laticifers might act as a putative storage of excess atmospheric carbon and that they can regulate levels of atmospheric isoprene gas (Hagel et al., 2008). Many of the other latex-containing plants are important to the global economy because poppy is a source of opium and the rubber tree is well known as being a source of natural rubber (Chow et al., 2007; Decker et al., 2000; Nawrot et al., 2007). Furthermore, lettuce latex contains several sesquiterpenoid lactones, phytoalexins lettucenin A and costunolide, which are all directly related to the bitter taste of lettuce (Sessa et al., 2000).

Recently, proteomics has developed into an efficient tool to study the abundance and distribution of proteins in an organism (Chen and Harmon, 2006). In addition, the expression profiles of different tissues have been analyzed and the identification and localization of individual proteins of interest have been accomplished using proteomic approaches. Thus far, two-dimensional gel electrophoresis (2-DE) has been routinely used for protein identification in numerous studies. However, low-abundance proteins and basic proteins normally present in plant tissues cannot be easily separated using the 2-DE approach. A recently developed multidimensional protein-identification technology (MudPIT) is unbiased and is an alternative high-throughput technology that permits the identification of a large number of proteins (Li and Assmann, 2000; Rose et al., 2004; Washburn et al., 2001).

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Recently, several proteomic studies of proteins found in phloem and xylem saps of various plant species have been published. These studies underline the physiological significance of proteomes in plant vascular systems (Aki et al., 2008; Alvarez et al., 2006; Buhtz et al., 2004; Djordjevic et al., 2007; Giavalisco et al., 2006; Kehr et al., 2005). However, only a few proteomic analyses have been carried out on the components of latex in a limited number of plants (including *Papaver somniferum* and *Hevea brasiliensis*) (Chow et al., 2007; Decker et al., 2000; Nessler et al., 1990). This lack of data arises from limited available information regarding the genomic sequence of latex-containing plants. The sticky and viscous nature of latex (arising from oxygen exposure of unidentified chemical components of the latex) makes its study more difficult.

To achieve a detailed understanding of the latex proteome, MudPIT has been used to identify proteins present in lettuce latex. The spectra from lettuce latex obtained using liquid chromatography/tandem mass spectrometry (LC–MS/MS) have been compared against a protein database constructed from the expressed sequence tag (EST) database of lettuce established in the protein databases of the University of California (Davis) (http://cgpdb.ucdavis.edu/database/Database_Description.html) and the National Center for Biotechnology Information (NCBI). In sum, 587 lettuce latex proteins have been identified including a major portion of plastidial and mitochondrial proteins, metabolic pathway proteins, and viral or plant pathogen-related proteins.

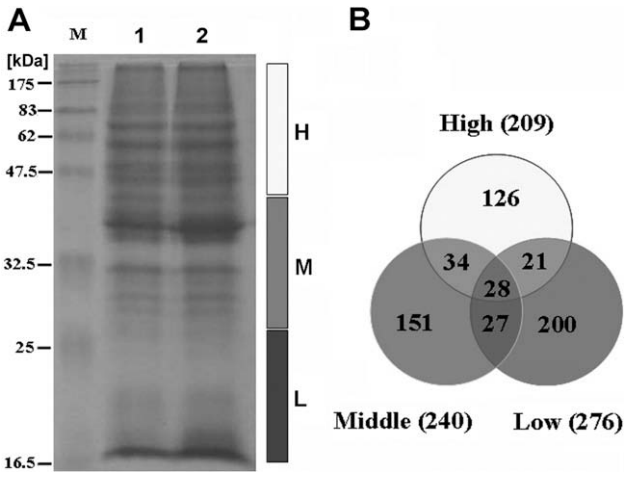

Fig. 1. SDS–PAGE of latex proteins from lettuce (*Lactuca sativa*). (A) SDS–PAGE analysis of the latex proteins from lettuce. Lanes: molecular marker, 1 (first experiment), 2 (second experiment); the molecular masses are indicated on the left. The one-dimensional gel of the latex proteins was excised into three sections; H (high), M (middle), and L (low). (B) Venn diagram of proteins identified in each gel section. A total of 587 proteins were identified by MudPIT analysis, and 138 proteins were found in at least two gel sections. Among them, 28 proteins were detected in all gel sections.

2. Results and discussion

2.1. Protein identification by MS/MS analysis

Protein identification inside lettuce latex by MS/MS analysis is limited due to its lack of genome-sequence information. Because protein identification relies exclusively on protein homology to other plant species it does not always give reliable data (Habermann et al., 2004). However, several previous studies have successfully applied the EST database of a plant species to protein identification by MS/MS analysis (Kim et al., 2003; Sheoran et al., 2007). In lettuce, more than 68,000 ESTs from various tissues (under The Compositae Genome Project) have been constructed from two distinct lettuce genotypes: cultivated lettuce (*Lactuca sativa L. cv. Salinas*) and wild lettuce (*Lactuca serriola L.*).

The isolated lettuce latex proteins were separated by electrophoresis using one-dimensional gel electrophoresis (1-DE) (Fig. 1A). Proteins on gels were stained for 30 min in Coomassie brilliant blue (CBB)-staining buffer. To enrich the number of proteins identified, the 1-DE gel was separated into high, middle, and low sections and then analyzed using the LC–MS/MS technique (Fig. 1A). MS/MS spectra obtained from the three gel sections were used to search for sequence similarity within a protein database constructed from the lettuce EST database and the lettuce protein database obtained from the NCBI. In total, 725 lettuce latex proteins from the three gel sections were identified in the lettuce EST database of the Compositae Genome Project Database (CGPDB) (596 proteins and 1,013 peptides) and the NCBI (129 proteins and 196 peptides) (Table S1 and Fig. 1B). After eliminating redundant proteins from the three gel sections, integration of all datasets indicated that 587 unique proteins (704 peptides) were lettuce latex proteins (Table S2). Interestingly, when the protein lists from the two different databases were compared, only one protein (deoxyhypusine synthase) was found to be common among them. This enzyme is involved in the posttranslational activation of the eukaryotic initiation factor 5A (eIF5A) (Ober and Hartmann, 1999). This result suggests that use of the lettuce EST database is a successful method for lettuce proteome analysis. In addition, it proves the effectiveness of using multiple databases for protein identification by MS/MS analysis.

The identified proteins had molecular masses ranging from 4.7 to 367 kDa (Table S2). Interestingly, 459 proteins (78%) had molecular masses between 20 and 30 kDa, indicating the presence of a large number of small molecules in lettuce latex. Organelles include several enzymes that permit them to synthesize many types of small molecules. Therefore, a large number of organellar proteins, such as plastidial and mitochondrial proteins, were detected in our analysis. The previously described latex proteome of the opium poppy had shown that the major latex proteins (comprising 50% of the latex proteins) had molecular masses of about 20 kDa (Decker et al., 2000; Nessler et al., 1990).

The isoelectric points (p*I*s) of identified latex proteins varied from 5.8 to 13 (Table S2). Compared to the number of acidic proteins (p*I* < 6), the number of basic proteins (p*I* > 8) was much greater and constituted 72% of the total proteins. Latex proteome from opium poppy contained either mostly acidic or neutral proteins. Specifically, only five proteins among the 98 proteins identified were basic (with p*I*s in the range of 8–9) in the opium study (Decker et al., 2000; Nessler et al., 1990). This result dramatically contrasts with the current data. The p*I* distribution in the lettuce proteome showed a normalized pattern within the p*I* range of 4–13 with a peak at the basic p*I* of 9–10. This result clearly shows the advantage of MudPIT compared to the classical 2D-gel analysis in the detection of basic proteins.

2.2. Functional classification of lettuce latex proteins

To categorize the latex proteins into functional classes, all latex proteins were converted into their respective *Arabidopsis* gene-identification (AGI) numbers derived from the *Arabidopsis* information resource (http://www.arabidopsis.org/) by sequence similarity (Table S2). The identified lettuce latex proteins were classified into functional classes using FunCat (Ruepp et al., 2004). More than half of the identified proteins consisted of five major protein classes: metabolism-related proteins (20%), cell rescue, defense, and virulence proteins (12%), proteins with binding function (10%), cellular transport proteins (8%), and proteins that control protein fate (8%).

The other groups contained proteins related to development (3%), protein synthesis (3%), and transcription (3%) (Fig. 2A). Unclassified proteins nevertheless comprised a major portion (29%) of the identified proteins (Fig. 2A). The representative latex proteins in each functional class are listed in Table 1 and are discussed individually below.

2.3. Subcellular localization of the identified latex proteins

To elucidate the putative cellular localization of lettuce latex proteins, the identified proteins were classified according to their subcellular localization using FunCat (Ruepp et al., 2004). Only 44% of the identified proteins were categorized by their subcellular localization (Fig. 2B). The proteins that were predicted to localize in the plastids and mitochondria were the largest groups accounting for 17% and 13% of all the identified proteins, respectively (Fig. 2B). In addition, proteins localized in the cytoplasm (6%) and the nucleus (4%) constituted the third and fourth major groups, respectively. This finding is in agreement with the previous latex proteome analysis of opium poppy which contained several proteins generally located in the plastids and mitochondria (Decker et al., 2000). These results suggest that latex cells are active metabolic sinks. The latex cells produce energy and chemicals within their mitochondria and plastids. To better understand the unknown 56% of the identified proteins for their subcellular localization, the putative localizations of the identified proteins were searched by several prediction programs (Table S2). They were obtained from SUBA II (http://www.plantenergy.uwa.edu.au/suba2/) (Heazlewood et al., 2007).

2.4. Metabolism-related proteins

The most interesting latex protein related to metabolism is hydroxymethylglutaryl coenzyme A synthase (EC 2.3.3.10) which was identified in *H. brasiliensis* and is involved in the early steps of rubber biosynthesis and functions as a regulatory enzyme in isoprenoid biosynthesis (Asawatreratanakul et al., 2003) (Table 1). The major components in lettuce latex were 15-oxalyl and 8-sulfate conjugates of the guaianolide sesquiterpene lactones, lactucin, deoxylactucin, and lactucopicrin (Sessa et al., 2000). Recently, the presence of rubber in lettuce latex has been reported (Bushman et al., 2006). Rubber molecules are synthesized in the cytoplasm of plant laticifer cells by a rubber transferase (EC 2.5.1.20) (Siler et al., 1997). The isoprenoid precursors of rubber are thought to be synthesized in the cytosol as part of the general mevalonic acid (MVA) pathway (Kush, 1994). The current latex proteome also comprises several enzymes related to isoprenoid biosynthesis including prenylcysteine oxidase, violaxanthin deepoxidase-related protein, gibberellin 2-oxidase, gibberellin 20-oxidase, and geranylgeranyl reductase.

Furthermore, other latex proteins such as fructose-1,6-bisphosphatase (EC 3.1.3.11) and triose-phosphate isomerase which are involved in glycolysis (EC 5.3.1.1) have been previously reported (Decker et al., 2000).

In addition, enzymes specifically related to antioxidation have been detected. Of these, glutamate-cysteine ligase (EC 6.3.2.2) and phosphomannomutase (EC 5.4.2.8) are well known for their roles in the plant defense response (Jez et al., 2004; Qian et al., 2007). Glutathione (EC 1.8.4.9) is another important intracellular antioxidant that is accumulated as a protective mechanism in response to different stress stimuli. The glutamate-cysteine ligase identified in the latex catalyzes the first step in glutathione biosynthesis and plays an important role in regulating the intracellular redox environment (Jez et al., 2004). Phosphomannomutase catalyzes the interconversion of mannose-6-phosphate and mannose-1-phosphate. A recent study provides genetic evidence for the involvement of phosphomannomutase in the biosynthesis of ascorbic acid in *Arabidopsis* (Qian et al., 2007).

The lettuce latex contains enzymes necessary for nitrogen assimilation and fatty acid synthesis. Glutamine synthetase (EC 6.3.1.2) is a key enzyme in nitrogen assimilation and is responsible for catalyzing the synthesis of glutamine from ammonium and glutamate (Ishiyama et al., 2004). Previously, an analysis of the transport fluids (xylem, phloem, and nodule exudates) from *Robinia pseudoacacia* showed that asparagine and glutamine were the major translocated nitrogenous solutes. These solutes are the key components of plant metabolism. *S*-Malonyltransferase (EC 2.3.1.39) is an enzyme required for the biosynthesis of fatty acids and lipoic acid in plant mitochondria (Gueguen et al., 2000). A previous investigation to decipher the mechanism of activation of malonate into malonyl-ACP in the pea leaf mitochondria showed for the first time that plant mitochondria contain a malonyl-CoA synthetase and a malonyl-CoA:ACP transacylase that are able to activate malonate into malonyl-ACP (Gueguen et al., 2000).

Three enzymes linked to ureide metabolism were detected in the latex. In ureide-transporting legumes, uricase (EC 1.7.7.3) plays a key role in the formation of allantoin. Uricase is a homotetramer of a 33 kDa polypeptide, nodulin-35, identified in a number of legumes. Uricase is localized in the peroxisomes of uninfected nodule cells. Allantoinase (EC 3.5.2.5) is a key enzyme for the biogenesis and degradation of these ureide compounds (Yang and Han, 2004). Urease (EC 3.5.1.5) is a nickel-containing urea hydrolase that catalyzes the hydrolysis of urea to ammonia and CO and is found in many microbes and plants. Moreover, urea is one of the most commonly used nitrogen fertilizers worldwide (Witte et al., 2005).

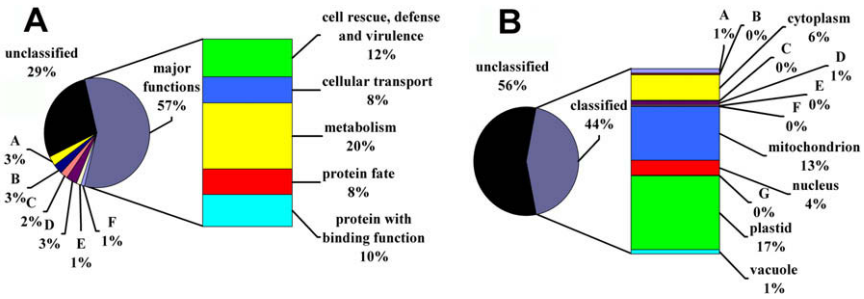

Fig. 2. Functional classification and subcellular localization of the 587 latex proteins identified. Each of the unique proteins was classified according to the FunCat groups (Ruepp et al., 2004). (A) Functional classification of the 587 latex proteins identified. A, transcription; B, protein synthesis; C, interaction with the environment; D, development; E, cellular communication, F, cell cycle and DNA processing. (B) Subcellular localization of the 587 latex proteins identified. A, plasma membrane; B, cell wall; C, cytoskeleton; D, endoplasmic reticulum; E, Golgi; F, intracellular transport vesicles; G, peroxisome.

Table 1
Representative latex proteins from the three major functional classes present in lettuce as identified by MudPIT analysis.

Sequence or Protein[a]	Functional description[b]	Matched org.[c]	Seq. count[d]	Seq. cov. (%)[e]	MW (Da)[f]	pI[g]
Metabolism						
JC432*	Alcohol dehydrogenase 1	LST	11	21	41335	6.9
AAF19790*	Fructose-1,6-bisphosphatase	LST	1	6	25192	5.3
QGJ4K08.yg.ab1_5	Geranylgeranyl reductase	GM	1	5	25336	11.1
BAB12443*	Gibberellin 2-oxidase 1	LST	1	4	38346	5.9
BAA37127*	Gibberellin 20-oxidase	LST	1	4	43498	7.2
QGG33P13.yg.ab1_2	Glutamate-cysteine ligase	ST	1	10	26716	9.8
AJLCQB*	Glutamine synthase	LST	3	11	39577	5.7
P23712*	Glutamine synthase	AT	1	4	39470	5.4
QGB18O20.yg.ab1_4	Histidinol dehydrogenase	AT	1	13	22600	5.3
QGC8I14.yg.ab1_1	Hydroxymethylglutaryl-CoA synthase	HB	1	7	26928	8.7
QGC8I14.yg.ab1_5	Hydroxymethylglutaryl-CoA synthase	HB	6	26	26779	8.8
QGD9A23.yg.ab1_5	Hydroxymethylglutaryl-CoA synthase	HB	1	6	26823	6.9
QGG12H18.yg.ab1_6	Phosphomannomutase	AT	1	6	27864	7.9
QGG16J11.yg.ab1_6	Prenylcysteine oxidase precursor	AT	2	11	27224	8.9
QGC26O10.yg.ab1_4	*S*-Malonyltransferase	AT	3	17	19788	8.5
QGE7H22.yg.ab1_2	Sarcosine oxidase family protein	AT	1	9	18545	8.9
QGE7H22.yg.ab1_3	Sarcosine oxidase family protein	AT	1	9	17366	9.6
QGC5D10.yg.ab1_6	SQD1 (sulfoquinovosyldiacylglycerol 1)	AT	1	5	27281	9.6
QGG6K01.yg.ab1_6	SQD2 (sulfoquinovosyldiacylglycerol 2)	AT	4	22	27465	7.8
AAB23371*	Triose-phosphate isomerase	LST	11	45	20540	5.4
AAB23372*	Triose-phosphate isomerase	LST	1	27	4674	4.7
QGC5N17.yg.ab1_1	Urease	ST	1	6	27851	9.4
QGC5N17.yg.ab1_4	Urease	ST	1	6	27449	9
QGF15I21.yg.ab1_3	Uricase	LJ	1	9	15037	6.5
QGI13N06.yg.ab1_1	Biolaxanthin de-epoxidase-related	AT	1	9	28283	7.7
Cell rescue, defense and virulence						
CAC01262*	19 kDa protein	TB	1	5	21424	7.3
YP_053237*	22 kDa protein	LR	1	3	22424	7.8
NP_848528*	25 K protein	ML	1	5	24776	8.8
YP_053236*	261 kDa protein	LR	2	1	262032	7
YP_053240*	37 kDa protein	LR	1	7	37731	8.2
NP_848530*	37 K protein	ML	1	4	37261	8
YP_053239*	48 kDa protein	LR	1	3	48264	8.3
AAG32648*	4a protein	LN	1	4	32499	4.7
YP_053238*	50 kDa protein	LR	1	4	50954	8.6
NP_848532*	55 K protein	ML	1	1	54586	9
AAU12865*	Coat protein	LB	1	5	44286	6.7
AAU12873*	Coat protein	ML	1	4	48546	8.3
BAD95904*	Coat protein	LB	1	3	44487	6.6
NP_619697*	Coat protein	LI	1	5	27770	6.4
AAL00992*	Disease resistance protein (CC–NBS–LRR class)	TC	1	9	19095	7.8
AAL00999*	Disease resistance protein (CC–NBS–LRR class)	TC	1	6	19718	8.9
AAP40918*	Disease resistance protein (CC–NBS–LRR class)	LP	1	8	20206	7.1
AAP45839*	Disease resistance protein (CC–NBS–LRR class)	HA	1	5	42732	6.4
AAQ72580*	Disease resistance protein (CC–NBS–LRR class)	LST	1	1	120851	6.2
AAR02571*	Disease resistance protein (CC–NBS–LRR class)	LST	2	6	57810	5.5
AAL00984*	Disease resistance protein (NBS–LRR class)	TC	1	11	19225	5.8
AAL01007*	Disease resistance protein (NBS–LRR class)	TC	1	15	18752	7.5
AAN87312*	Disease resistance protein (NBS–LRR class)	LST	1	12	10806	5.2
AAQ72572*	Disease resistance protein (NBS–LRR class)	LST	1	2	102536	6.2
AAQ72577*	Disease resistance protein (NBS–LRR class)	LST	1	1	84624	6.2
AAQ72578*	Disease resistance protein (NBS–LRR class)	LST	2	2	146845	7
AAQ73104*	Disease resistance protein (NBS–LRR class)	LST	1	3	47922	6.2
AAQ73148*	Disease resistance protein (NBS–LRR class)	LST	1	4	46849	6.2
AAQ73164*	Disease resistance protein (NBS–LRR class)	LSL	1	3	46950	6.4
AAQ73167*	Disease resistance protein (NBS–LRR class)	LSL	3	14	47037	6.3
AAR02572*	Disease resistance protein (NBS–LRR class)	LST	2	2	218202	6.8
T30558*	Disease resistance protein (NBS–LRR class)	LST	1	1	207838	6.3
T30559*	Disease resistance protein (NBS–LRR class)	LST	2	2	195037	5.4
T30560*	Disease resistance protein (NBS–LRR class)	LST	2	2	199290	6.2
AAN87302*	Disease resistance protein (TIR–NBS–LRR class)	LST	1	40	10383	7.4
AAN87303	Disease resistance protein (TIR–NBS–LRR class)	LST	3	15	10647	6.3
AAN87304	Disease resistance protein (TIR–NBS–LRR class)	LST	1	15	10790	5.8
NP_619698*	Duplicated coat protein	LI	2	5	51962	8.3
AAP86603*	LSP1 (loss of susceptibility to Potyviruses)	LST	1	6	21968	5.7
P89876*	Genome polyprotein	SA	2	1	367625	8
P31999*	Genome polyprotein	LM	1	0	367572	7.8
NP_619695*	Heat-shock protein	LI	1	2	62277	7.8
BAC16226*	L protein	LB	2	2	232111	8.4
AAL01028*	NBS/LRR resistance protein-like protein	TC	1	3	28062	6.9
AAN87324*	NBS/LRR resistance protein-like protein	HA	1	14	18587	5.1
CAG34090*	Nucleocapsid protein	LN	2	6	50684	8.2
AAF19789*	Peptide methionine sulfoxide reductase	LST	1	4	28842	8.4

(*continued on next page*)

Table 1 (*continued*)

Sequence or Protein[a]	Functional description[b]	Matched org.[c]	Seq. count[d]	Seq. cov. (%)[e]	MW (Da)[f]	pI[g]
CAB01623*	Polyprotein	LM	1	4	38089	5.5
CAD56235*	Polyprotein	LM	2	7	19072	9.3
CAG34091*	Putative polymerase	LN	1	1	236581	7.9
NP_848527*	Putative RNA polymerase	ML	1	1	262633	6.9
NP_733943*	Replicase	LI	2	2	228449	7.7
AAQ73151*	Resistance protein RGC2	LST	1	3	55686	5.4
AAP45842*	RPS2 (resistant to p. syringae 2)	HA	1	4	44881	6.5
AAQ72575*	RPS2 (resistant to p. syringae 2)	LST	1	2	84756	5.6
AAQ73133*	RPS2 (resistant to p. syringae 2)	LSL	2	3	56296	6.4
QGG23K04.yg.ab1_2	Ferredoxin oxidoreductase	AT	1	6	24997	10
QGG23K04.yg.ab1_6	Ferredoxin oxidoreductase	AT	1	6	26779	7.5
QGD7L06.yg.ab1_1	Ferredoxin thioredoxin reductase beta chain	ST	1	10	26568	9.1
QGD7L06.yg.ab1_5	Ferredoxin thioredoxin reductase beta chain	ST	1	5	27662	8.4
Protein fate						
QGG12A05.yg.ab1_1	CAND1	AT	1	5	27179	9.7
QGJ1K24.yg.ab1_1	COX15 (cytochrome c oxidase 15)	AT	1	14	23357	10.2
QGF9C20.yg.ab1_1	Subunit NDH-N of NAD(P)H	AT	1	5	21383	9.2
QGG20D23.yg.ab1_1	MAX2 (more axillary branches 2)	AT	1	4	25538	11.1
QGF17P02.yg.ab1_6	PBG1 (20S proteasome beta subunit G1)	AT	4	14	27376	6.8
QGG35K15.yg.ab1_5	TIC110 (chloroplast import apparatus 110)	AT	2	11	27591	5.5
QGJ12B18.yg.ab1_5	TIC21 (chloroplast import apparatus 21)	AT	1	8	22604	9.8

Matched org., Matched organism; Seq. count, Sequence count; Seq. cov., Sequence coverage; MW, Molecular weight; p*I*, Isoelectric point3.

[a] Accession number of EST sequences from the lettuce EST database and from the NCBI, respectively. Lettuce proteins derived from the NCBI are indicated by asterisk.

[b] Although some proteins might belong to several functional classes, each identified protein has a single function in this analysis for simplification. The partial amino acid sequences obtained are provided in Table S1 and the detailed information on other latex proteins identified can be accessed from Table S2.

[c] AT, *Arabidopsis thaliana*; GM, *Glycine max*, HA, *Helianthus annuus*; HV, *Hevea brasiliensis*; LP, *Lactuca perennis*; LSL, *Lactuca saligna*; LST, *Lactuca sativa*; LB, *Lettuce big-vein virus*; LI, *Lettuce infectious yellows virus*; LM, *Lettuce mosaic virus*; LN, *Lettuce necrotic yellows virus*; LR, *Lettuce ring-necrosis virus*; LJ, *Lotus japonicus*; OS, *Oryza sativa*; ST, *Solanum tuberosum*; SA, *Staphylococcus aureus, strain USA300*; TC, *Theobroma cacao*; TB, *Tomato bushy stunt virus*. MS/MS spectra obtained from the LC–MS/MS analyses were searched against two independent databases using Bioworks™ v. 3.1.

[d] The number of peptides that were matched to the obtained protein in MudPIT analysis.

[e] The percentage of amino acids in the given peptide matched to the identified protein.

[f] Molecular weight of identified protein.

[g] Isoelectric point of identified protein.

* Accession number of lettuce proteins in the NCBL.

The lettuce latex, furthermore, contains alcohol dehydrogenase, which is a type of glycolytic enzyme capable of producing ethanol in response to hypoxic or anoxic conditions. This phenomenon is accepted as the main adaptation of plants to anaerobic stresses. A previous study has shown the presence of alcohol dehydrogenase in the vascular cambium of trees (Kimmerer and Stringer, 1988).

Two proteins, gibberellin 2-oxidase (EC 1.14.11.13) and gibberellin 20-oxidase (EC 1.14.11.15), have been identified as enzymes involved in gibberellin biosynthesis. Gibberellin hormones participate in the regulation of many important processes in higher plants including the development of seeds and seedlings (Laule et al., 2003).

Two sulfoquinovosyldiacylglycerol (SQD1 and SQD2) (EC 3.13.1.1) containing proteins that catalyze sulfolipid biosynthesis were detected in the latex. Sulfolipid is a type of glycolipid that functions as a structural lipid in the photosynthetic membranes of seed plants (Mulichak et al., 1999). The synthesis of sulfoquinovosyldiacylglycerol is not essential under normal growth conditions, but it is postulated that it becomes important when phosphate availability is limited (Mulichak et al., 1999; Yu et al., 2002).

Sarcosine oxidase (SOX) (EC 1.5.3.1) has also been found in the latex. Mammals and various soil bacteria contain sarcosine oxidases, which catalyze the oxidation of sarcosine, other *N*-methyl amino acids, and L-pipecolate. This catalysis yields formaldehyde, glycine, and H_2O_2, respectively. A previous study on *Arabidopsis* has shown that this enzyme is imported into the peroxisomes and can oxidize sarcosine in vivo (Goyer et al., 2004). However, sarcosine oxidase of *Arabidopsis* is expressed at low levels throughout the plant. In addition, the latex contains histidinol dehydrogenase (EC 1.1.1.23), which catalyzes the terminal step in the biosynthesis of histidine in bacteria, fungi, and plants. This protein is mainly involved in the four-electron oxidation of L-histidinol to histidine (Muralla et al., 2007). A recent study has shown the importance of histidine biosynthesis for the growth and development of plants using a combination of forward and reverse genetic approaches (Muralla et al., 2007). The current proteome results provide valuable insights into several metabolic enzymes present in the latex.

2.5. Proteins related to cell rescue, defense, and virulence

The lettuce latex proteins assigned to the functional class of cell rescue, defense, and virulence mostly belonged to the lettuce disease resistance protein family and several lettuce viral proteins (Table 1).

The lettuce viral proteins identified in the latex were isolated from lettuce mosaic virus (LMV), mirafiori lettuce big-vein virus (MLBVV), lettuce big-vein virus (LBVV), lettuce infectious yellows virus (LIYV), lettuce ring necrosis virus (LRNV), and lettuce necrotic yellows virus (LNYV). Most of the lettuce viruses causing diseases are transmitted in a nonpersistent manner by aphids (Nebreda et al., 2004). LMV is one of the most common pathogens of lettuce. LMV belongs to the genus Potyvirus of the family *Potyviridae* (Peypelut et al., 2004). MLBVV is assigned to the genus Ophiovirus, with filamentous, kinked, circularized virions (Navarro et al., 2005). LBVV belongs to the genus *Varicosavirus* and has been purified from big-vein affected lettuce. These viruses cause severe leaf deformations and growth reduction and cause remarkable economic losses. LIYV belongs to the genus *Crinivirus* of the family *Closteroviridae* (Yeh et al., 2000) and its complete genomic sequence is available (Klaassen et al., 1995). Infections due to viruses of the family *Closteroviridae* are mostly restricted to the phloem tissues of lettuce, and the viruses are essentially dependent on their phloem-feeding insect vectors for plant-to-plant transmission. Ring necrosis is a serious disease of lettuce which results in necrotic rings and ring patterns in leaves. This disease is caused by LRNV, which is transmitted by the chytrid fungus *Olpidium brassicae* as a vector from the soil (Huijberts, 1996). LNYV is the type member of the genus

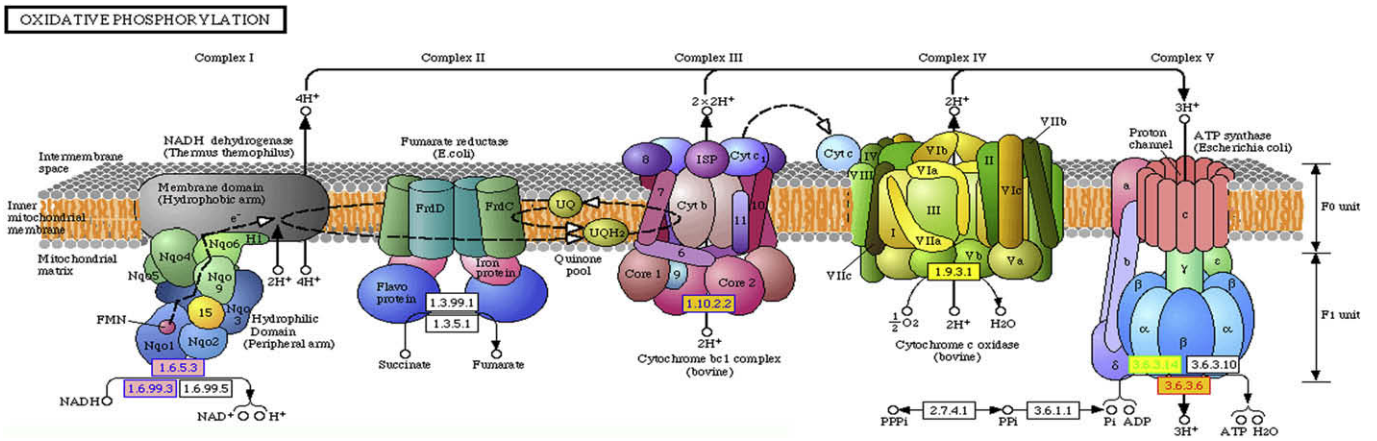

Fig. 3. The identified lettuce latex proteins involved in oxidative phosphorylation derived from KEGG pathway implemented in Blast2go. Lettuce latex proteins were converted to the corresponding *Arabidopsis* locus and were mapped on the corresponding KEGG pathway. The colored box indicates the corresponding EC number of identified proteins. Detailed list of proteins in each metabolic pathway can be found in Table 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

cytorhabdovirus of the family *Rhabdoviridae* and LNYV causes yellow bands and necrotic areas in the leaves (Dietzgen et al., 2006).

Higher plants have evolved sophisticated defense mechanisms to counteract biotic and abiotic stresses. Specifically, the interactions between plants and pathogens are regulated by specific reciprocal interactions between the *avr* (avirulence) gene loci of the pathogen and the alleles of the corresponding disease resistance (*R*) loci of the plant (Dangl and Jones, 2001). Hitherto, many *R* genes have been identified from many plants by several groups. Among them, the largest class of disease resistance gene families identified encodes proteins containing highly duplicated nucleotide-binding site leucine-rich repeats (NBS–LRRs). These NBS–LRR proteins are regulated by the members of multiprotein complexes and they detect the binding of the avirulence protein to one or more members of the complex (Dangl and Jones, 2001). Several studies have shown that many disease resistance proteins are often clustered together in plant genomes (Meyers et al., 2003). In lettuce, a large number of RGC2 genes have been identified from seven genotypes of three *Lactuca* species (Kuang et al., 2004). In this study, 36 resistance proteins belonging to the family of NBS/LRR resistance proteins and resistance-protein RGC2 have been identified. In addition to the RGC2 proteins, several proteins found in the latex were related to the defense mechanism of plants. One is a putative LSP1 (loss-of-susceptibility to Potyviruses) protein that plays a role in the induction of eIF(iso)4E during Potyvirus infection (Lellis et al., 2002).

Reactive oxygen species (ROS) are produced in response to a wide range of biotic and abiotic stresses. Several antioxidant enzymes have been identified in the latex (Table 1). Among them, the plastid-targeted peptide, methionine sulfoxide reductase (pPMSR), can rescue enzymatic activity when ROS such as hydroxyl radicals and superoxide ions disrupt the methionine residues of many enzymes in cells. Thioredoxins are complex systems regulating cellular redox balance in plants. Two plastidial proteins, ferredoxin oxidoreductase (EC 1.2.7.5) (Kohchi et al., 2001) and the catalytic beta chain of ferredoxin thioredoxin reductase, in addition to mitochondrial NADP adrenodoxin-like ferredoxin reductase of the ferredoxin–thioredoxin system have been detected. All the three enzymes play important roles in the reduction and oxidation of thioredoxin. These data suggest that this oxidative stress repair enzyme plays putative roles in the latex tissues. It is apparent that the latex contains defense proteins against lettuce viruses.

Table 2
The 14 identified lettuce latex proteins involved in oxidative phosphorylation within mitochondria. The identified lettuce latex proteins were converted to the corresponding *Arabidopsis* locus to map their corresponding EC numbers implemented in Blast2Go.

EST sequence ID	Functional description	AT locus	Sequence count	Sequence coverage (%)	Molecular weight	*pI*
EC 1.9.3.1: cytochrome-c oxidase						
QGB8E01.yg.ab1_2	Cytochrome c oxidase assembly protein	AT1G02410	1	10	15145	10
QGJ1K24.yg.ab1_1	Cytochrome oxidase 15	AT5G56090	1	14	23357	10.2
EC 3.6.3.14: H+ transporting two sector ATPase						
QGF12N21.yg.ab1_5	DET3 (DE-ETIOLATED 3)	AT1G12840	2	10	26230	6
QGC17F02.yg.ab1_6	Vacuolar ATP synthase subunit H	AT3G42050	5	24	27802	9.3
QGB8M17.yg.ab1_6	Vacuolar ATP synthase subunit D	AT3G58730	4	12	28560	10
QGA6H24.yg.ab1_4	Vacuolar ATP synthase subunit F	AT4G02620	2	16	23595	6.7
QGE11K18.yg.ab1_5	ATP synthase delta chain	AT4G09650	1	7	23516	9.1
QGB15I17.yg.ab1_5	ATP synthase delta chain	AT5G47030	1	10	17533	9.7
EC 1.10.2.2: ubiquino cytochrome-c reductase						
QGA8B24.yg.ab1_6	Ubiquinol-cytochrome C reductase	AT3G52730	1	7	20472	9.5
EC 3.6.3.6: H+ exporting ATPase						
QGB8M17.yg.ab1_6	Vacuolar ATP synthase subunit D	AT3G58730	4	12	28560	10
QGA6H24.yg.ab1_4	Vacuolar ATP synthase subunit F	AT4G02620	2	16	23595	6.7
EC 1.6.5.3: NADH dehydrogenase (ubiquinone)						
QGJ9J19.yg.ab1_5	NADH-ubiquinone oxidoreductase B18 subunit	AT2G02050	1	8	22459	9.1
QGC25G04.yg.ab1_4	NADH dehydrogenase	AT5G37510	1	9	26286	9.1
QGA17N13.yg.ab1_5	NADH-ubiquinone oxidoreductase B8 subunit	AT5G47890	1	9	18160	9.6

2.6. Proteins associated with protein fate

Several proteins associated with ubiquitin proteasome-mediated protein degradation were also detected in the latex. They were (i) putative *Arabidopsis* CAND1 (an unmodified CUL1-interacting protein involved in multiple developmental pathways) (Feng et al., 2004), (ii) more axillary branches 2 (MAX2, a type of ubiquitin-protein ligase) (Stirnberg et al., 2007), and (iii) the 20S proteasome beta subunit G1 (a ubiquitin family protein). These results lead to the conclusion that the ubiquitin proteasome system is present in the latex and allows plants to effectively control their growth and development in response to environmental changes (Schwechheimer and Calderon Villalobos, 2004).

2.7. Organellar proteins related to various metabolites

One of the interesting results in our study is that a large number of lettuce latex proteins are predicted to localize in organelles. To get a better insight of identified proteins in a specific metabolic pathway, Blast2go program was used to map the identified lettuce latex proteins on the corresponding metabolic pathway following KEGG pathway database (Conesa and Gotz, 2008; Kanehisa et al., 2002). Of those, 14 proteins composing main protein complexes of the oxidative phosphorylation in mitochondria, such as cytochrome oxidase, ATP synthase, and NADH dehydrogenase, were detected in the lettuce latex sap (Fig. 3 and Table 2). Oxidative phosphorylation is the most important part of the metabolic pathways which produces adenosine triphosphate (ATP) by utilization of energy, and it also produces reactive oxygen species, such as hydrogen peroxide and superoxide. In conclusion, the fact that many organellar proteins are present in the latex sap is well correlated with the presence of numerous metabolites which are derived from organelles, such as plastids and mitochondria. Previous proteome analyses of opium poppy, *Chelidonium majus*, and *Calotropis procera* supported that specialized latex metabolites were included in laticifers, but their protein compositions are quite variable depending on the species (Decker et al., 2000; Freitas et al., 2007; Nawrot et al., 2007).

3. Concluding remarks

The main purpose of this study was to give the first comprehensive overview of the latex proteins of lettuce based on the available lettuce EST database. Successful application of the lettuce EST and NCBI databases led to the identification of a large number of lettuce latex proteins. The identified latex proteins were different from those of the two major vascular systems (phloem and xylem) in various plant species. This indicates that the unique latex proteins have unidentified roles in not only plant transport systems but also plant defense mechanisms. In addition, a large number of latex proteins have been confirmed to be organellar proteins localized in plastids and mitochondria. Surprisingly, RGC2 proteins and lettuce viral proteins were identified in lettuce latex which indicates the latex's special role in plant defense against pathogens. This is the first report to indicate that the lettuce latex contains many viral and defense-related proteins. The resulting proteome data will facilitate future research of latex related to defense mechanisms in higher plants.

4. Experimental

4.1. General experimental procedures

Lettuce (*Lactuca sativa L.*) seeds were kindly provided by Dr. Ju-Hyeon Kim (Gyeongsang National University). Plants were grown in a greenhouse (18–30 °C) under natural light conditions during the spring season. MudPIT has been used to identify the proteins present in lettuce latex. All solvents used for in-gel digestion and peptide sample preparation were of high-performance liquid chromatography (HPLC) grade. Peptides eluted from the capillary column were electrosprayed onto an LTQ linear ion-trap mass spectrometer (ThermoFinnigan, Waltham, MA). Bioworks™ v. 3.1 (Beckman Coulter Inc., Fullerton, CA, USA) was used to filter the search results.

4.2. Lettuce latex sampling

The latex sap was collected from 3-month old *L. sativa* plants. Stems of lettuces were pricked using a needle. The first drop of sap from the stem was removed with H_2O and dabbed with filter paper to avoid contamination. Subsequently, droplets of latex flowing from the holes of the lettuce stems were collected with a hand-held pipette. The exuding latex was immediately expelled into PBS solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, pH 8.0) placed on ice, frozen in liquid N_2, and stored at −80 °C for further analysis.

4.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Isolated latex proteins were resuspended in 2× SDS-loading buffer containing 4 M urea by boiling for 5 min. Samples were loaded on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel and were run for 45 min at 100 V. Proteins on gels were visualized after staining for 30 min in Coomassie brilliant blue (CBB) -staining buffer (50% MeOH, 10% glacial AcOH, 40% H_2O, and 0.5 g/L CBB). Gels were destained thoroughly in a destaining buffer (MeOH:glac.AcOH:H_2O (3:1:6) v/v). Three gels were prepared from three different protein extractions.

4.4. In-gel digestion and peptide sample preparation

Protein bands were excised from CBB-stained gel. Subsequently, the excised bands were washed three times with a 1:1 (v/v) solution of CH_3CN/deionized H_2O for 10 min and dehydrated with CH_3CN. The bands were finally washed with 1:1 (v/v) solution of CH_3CN:100 mM ammonium bicarbonate and dried using a Speed-Vac. Proteins separated in the gel pieces were reduced using 10 mM tris(2-carboxyethyl)phosphine in 0.1 M ammonium bicarbonate at 56 °C for 45 min and then alkylated with 55 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 30 min. The washing step mentioned above was repeated on the alkylated sample. After the washing step, the gel pieces were dried and soaked in sequencing-grade trypsin solution (500 ng) and placed on ice for 45 min. Subsequently, gel pieces were immersed in 100 μL of 50 mM ammonium bicarbonate (pH 8.0) at 37 °C for 14–18 h. The resulting peptides were extracted sequentially with CH_3CN:20 mM ammonium bicarbonate (45:55, v/v), CH_3CN:0.5% CF_3CO_2H in H_2O (45:55, v/v) (TFA), and CH_3CN:0.25% TFA in H_2O (75:25, v/v), with agitation for 20 min each. The extracts containing the tryptic peptides were pooled together and evaporated under vacuum.

4.5. Micro-LC/LC–MS/MS analysis

A single-phase microcapillary column was constructed with a 100 μm inner diameter (i.d.) fused-silica capillary tubing, which was pulled to a 5 μm i.d. tip using a CO_2 laser puller (P-2000, Sutter Instruments, Novato, CA). The capillary column was packed sequentially with 7 cm of 5 μm i.d. Polaris C18-A (Metachem, West Warwick, RI) and then 3 cm of 5 μm i.d. Partisphere strong cation exchanger (Whatman, Florham Park, NJ), followed by another 3 cm

of Polaris C18-A using a homemade high-pressure column loader. The columns were equilibrated with CH_3CN:0.1% HCO_2H in H_2O (5:95. v/v) solution and about 10–25 μg of lettuce latex protein digest was directly loaded onto the capillary column. The buffer solutions used to separate the protein digests were buffer A, CH_3CN:0.1% HCO_2H in H_2O (80:20, v/v) (buffer B), and 500 mM $(NH_4)OAc$ in CH_3CN:0.1% HCO_2H in H_2O (5:95, v/v) (buffer C). Six steps of strong cation exchange liquid chromatography reversed-phase HPLC peptide separation were conducted. Step 1 consisted of a 100 min gradient from 0% to 100% buffer B. Steps 2–5 had the following profiles: 3 min of 100% buffer A, 2 min of buffer C, a 10 min gradient from 0% to 15% buffer B, and a 97 min gradient from 15% to 45% buffer B. The 2 min buffer C percentages were 10%, 20%, 40%, 60%, and 100%. Peptides eluted from the capillary column were electrosprayed onto an LTQ linear ion-trap mass spectrometer (ThermoFinnigan, Waltham, MA) by applying a distal 2.4 kV spray voltage. A cycle, consisting of one full scan (400–1400 m/z) followed by nine data-dependent MS/MS scans at 35% normalized collision energy, was repeated throughout the LC separation.

4.6. Database search

MS/MS spectra obtained from the LC–MS/MS analyses were searched against a database containing lettuce EST sequences from the CGPDB (http://cgpdb.ucdavis.edu/database/Database_Description.html) and a lettuce protein database deposited in the NCBI (http://www.ncbi.nlm.nih.gov/). Tandem mass spectra were extracted by Bioworks™ v. 3.1. Charge state deconvolution and deisotoping were not performed. Sequest was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 3.0 Da. Iodoacetamide derivative of cysteine was specified in Sequest as a fixed modification (cysteines +57). Oxidation of methionine was specified in Sequest searches as a variable modification (methionine +16) and the maximum number of modifications that were allowed per peptide was three (the maximum number of modifications per type was five). The number of missed cleavage sites for trypsin digestion was two. Proteins that contained similar peptides and that could not be differentiated based on the MS/MS analysis alone were grouped to satisfy the principles of parsimony. Bioworks v. 3.1 was used to filter the search results and the following Xcorr values and delta Cn value of 0.08 were applied to different charge states of peptides: 1.8 for singly charged peptides, 2.5 for doubly charged peptides and 3.5 for triply charged peptides.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.03.004.

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