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Proteomics Approach to Identify Unique Xylem Sap Proteins in Pierce's Disease-Tolerant *Vitis* Species

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Abstract Pierce's disease (PD) is a destructive bacterial disease of grapes caused by *Xylella fastidiosa* which is xylem-confined. The tolerance level to this disease varies among *Vitis* species. Our research was aimed at identifying unique xylem sap proteins present in PD-tolerant *Vitis* species. The results showed wide variation in the xylem sap protein composition, where a set of polypeptides with p*I* between 4.5 and 4.7 and M_r of 31 kDa were present in abundant amount in muscadine (*Vitis rotundifolia*, PD-tolerant), in reduced levels in Florida hybrid bunch (*Vitis* spp., PD-tolerant) and absent in bunch grapes (*Vitis vinifera*, PD-susceptible). Liquid chromatography/mass spectrometry/mass spectrometry analysis of these proteins revealed their similarity to β-1, 3-glucanase, peroxidase, and a subunit of oxygen-evolving enhancer protein 1, which are known to play role in defense and oxygen generation. In addition, the amount of free amino acids and soluble sugars was found to be significantly lower in xylem sap of muscadine genotypes compared to *V. vinifera* genotypes, indicating that the higher nutritional value of bunch grape sap may be more suitable for *Xylella* growth. These data suggest that the presence of these unique proteins in xylem sap is vital for PD tolerance in muscadine and Florida hybrid bunch grapes.

Keywords Grape · Pierce's disease · Differential protein expression · Xylem sap

Abbreviations

LC/MS/MS	Liquid chromatography/mass spectrometry/mass spectrometry
2-D	2-Dimensional electrophoresis

Introduction

Commercial grape cultivation is dominated by bunch (*Vitis vinifera* L., *Vitis labrusca* L., and other *Vitis* spp.) and hybrid bunch (*Vitis* spp.) grape genotypes, while muscadine grape

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(*Vitis rotundifolia*) genotypes are underutilized. Muscadine grape species are native to southeastern USA [1] and are more tolerant to most diseases including Pierce's disease (PD) than bunch grapes [2]. *V. vinifera* cultivars are highly susceptible to PD [3] while Florida hybrid bunch grape cultivars, which were developed through hybridization of local grape species with the table wine group, *V. vinifera* [4], are also tolerant to PD. But, their tolerance level varies compared to muscadine as the hybrids contain *V. vinifera*, a PD-susceptible species in their parentage. PD is caused by *Xylella fastidiosa* which is vectored by the glassy-winged sharp shooter and thrives in the xylem of grapevines [5]. Through colonization of xylem vessels, *X. fastidiosa* causes vessel clogging leading to wilting of the plant [6]. Xylem sap is known to contain various amino acids, sugars, organic acids, inorganic ions, proteins, and low concentration of organic compounds essential to support bacterial growth [7, 8].

Amino acids and organic acids are predominant organic compounds in xylem fluid of many woody species [9, 10]. The chemistry of xylem fluid is not fixed and can vary with temperature, time of year, light conditions, water stress, and soil nutrient status [8, 10]. Xylem sap is also known to contain proteins in low concentrations [7, 11, 12] and constitutes an environment in which pathogens can grow leading to vessel clogging and eventual death of the grapevine. Resistance and susceptibility of grapevines to PD could be determined by the interactions occurring within the xylem vessels between the *Xylella* and xylem sap components.

In both compatible (susceptible) and incompatible (resistance) plant–microbe interactions, plants respond by secreting their own set of proteins [13]. These proteins may either cause direct damage to invaders or play a protective role through inhibition of cell wall-degrading enzymes secreted by pathogens [14] or through oxygen sequestering to maintain the level of oxygen in xylem sap, which may be altered due to pathogen activity. Recent studies have shown that xylem sap proteins of broccoli, rape, pumpkin, cucumber, and tomato share homologies with several pathogen-related proteins including glycine-rich proteins, peroxidase-like proteins, chitinase-like proteins, serine protease-like proteins, aspartyl proteases, and lipid transfer-like proteins which are active in repair and defense reactions of the plant [15]. In tomato, it has been shown that xylem sap protein patterns change in response to infection by pathogenic fungi, and some of the proteins were identified as pathogenesis-related proteins [11, 12]. In addition, appearance of unique proteins has been documented in xylem sap during development of diseases affecting the vascular system [16]. Hence, xylem sap of infected plants may be a rich information source regarding molecular interaction underlying several plant diseases [13, 17].

While the importance of xylem sap proteins in other crops has been established, very little information is available on *Vitis* xylem sap proteins and their functions. To obtain a more comprehensive overview of the grape xylem chemistry, we undertook this study of analyzing xylem sap protein profiles of the *Vitis* species. This study will pave way towards further understanding the nature and function of *Vitis* xylem sap proteins/peptides and their role in plant defense and nutrition.

Materials and Methods

Plant Material The PD-tolerant grapevines belonging to the *V. rotundifolia* (cvs. Dixieland, Cowart, Noble, Alachua, African Queen, Black Beauty, Scuppernong, Pride, Regale, Higgins, Scarlet, Sweet Jenny, Senoia, Carlos, and Sterling) and Florida hybrid bunch (cvs. Lake Emerald, Blue Lake, and Orlando Seedless) grape genotypes grown at the vineyard of Center for Viticulture and Small Fruit Research, Florida A&M University, Tallahassee, FL,

USA were used in this study. Highly PD-susceptible *V. vinifera* genotypes (cvs. Barbara, Ruby Cabernet, Sauvignon Blanc, Chenin Blanc, Fiesta, Napa Gammy, Zinfandel, Petite Sarah, Merlot, Pinot Noir, Flame Seedless, JS2605, and Chardonnay) were grown in a screened cage to protect them from insects (Glossy Wing sharp shooters) that spread Pierce's disease and were used as "healthy" control plants. These grape plants were obtained from Sonoma Grapevines, Inc, Santa Rosa, CA, USA.

Xylem Sap Collection Phloem tissue from selected stem (spring growth) portions was peeled off using a sharp blade (to avoid contamination from phloem proteins), and sap from the peeled stem portion was collected by cutting the stem using a sharp clipper. The cut stem was thoroughly washed with water before collecting sap to avoid any possible contamination from phloem sap and other cellular compartments. Since *Xylella* multiplication is known to be high during the spring season, xylem sap was collected on alternative days for 3 days in the spring of 2007. Recovery of sap was found to be higher during early morning hours; therefore, sap collections were made during these hours. The sap was collected into sterile 50 ml Falcon tubes covered with parafilm to prevent contamination. The collected sap was brought to the lab on ice, divided into 20 ml aliquots, and freeze-dried. The dried material was reconstituted in rehydration buffer [8 M urea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, 50 mM dithiothreitol (DTT), and 2% ampholines (pH 3.0 to 10)] and used for two-dimensional polyacrylamide gel electrophoresis (2-D PAGE).

Confirmation of X. fastidiosa Infestation In order to confirm the presence of *Xylella* in xylem sap, one-dimensional PAGE was performed following the procedure described by Vasanthaiah et al. [18]. Equal amounts of freeze-dried xylem sap, *Xylella*, and blank PD3 broth used to grow *Xylella* were separated on the gel. Electrophoresis was carried out at a constant current of 30 mA, and the samples were silver-stained for visualization as described below.

α-Amino Nitrogen and Soluble Sugars Analysis An aliquot (100 ml) of the xylem sap was used for free amino acids and soluble sugars analysis to determine quantitative differences among *Vitis* species. α-Amino nitrogen analysis of xylem sap was carried out according to the procedure of Yemm and Cocking [19] and sugar content was determined using the method of Yemm and Willis [20].

Two-Dimensional Polyacrylamide Gel Electrophoresis The 2-D PAGE was performed on immobilized pH gradient (IPG) strips (pH 3 to10) on a BioRad Protean Cell following the manufacturer's instructions (BioRad Laboratories, Inc. Hercules, CA, USA). The freeze-dried xylem sap collected over different days (1, 3, and 5 days) were dissolved in rehydration buffer, and the IGP strips were soaked in this protein solution for 18 h and subjected to isoelectricfocusing (IEF) for a total of 20,000 V h. After IEF, the IPG strips were equilibrated for 10 min in equilibration buffer I [6 M urea, 0.375 M Tris–HCl, pH 8.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, 2% (*w*/*v*) DTT]. The IPG strips were then re-equilibrated with equilibration buffer II for another 10 min [6 M urea, 0.375 M Tris–HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% (*w*/*v*) iodoacetamide] and subjected to SDS-PAGE in the second dimension on 8% to 16% criterion gradient Tris–HCl pre-cast gels (BioRad Laboratories, Inc.).

Silver Staining The protein bands were visualized by silver staining following the method of Sambrook et al. [21]. Following electrophoresis, the gels were fixed in acetic acid, methanol,

and formaldehyde (50%:49.4%:0.6% *v*/*v*/*v*) for 45 to 60 min. The gels were then washed with methanol (50% *v*/*v*) for 15 min followed by distilled water. The gels were incubated in silver nitrite (0.15% *w*/*v*) for 30 to 45 min followed by washing with distilled water. The gels were then developed in 0.04% formaldehyde in 2% (*w*/*v*) sodium carbonate. The reaction was stopped after the desired intensity of the bands was obtained with 5% (*v*/*v*) acetic acid.

Mass Spectroscopy, Database Searches, and Sequence Analysis Xylem sap proteins unique to PD-tolerant muscadine genotypes were identified by comparing the 2-D PAGE profiles of the three *Vitis* species. Based on this comparison, ten protein spots that are unique to PD-tolerant *Vitis* species were selected for amino acid sequencing. Two polypeptide spots, B and C from box 1 (indicated by vertical arrows); seven spots, G, H, J, L, O, P, and Q from box 2; and one spot, C from box 3 (Fig. 2), were selected for sequencing. These proteins spots were selected because they were found to be unique among the PD-tolerant *Vitis* species used in the study. The protein spots were excised with a clean razor blade, destained, dehydrated, and subjected to trypsin digestion. Capillary RP-HPLC separation of protein digests (desalted with a PepMap C18 cartridge) was performed using a PepMap C18 column (15 cm×75 μm i.d.) and Ultimate Capillary HPLC System (LC Packings, San Francisco, CA, USA). A linear gradient of 5% to 40% (*v*/*v*) acetonitrile for 25 min at 200 nl min^{-1} was used for separation. Tandem mass spectrometric analysis was performed online using a hybrid quadrupole time-of-flight instrument (QSTAR XL hybrid liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS)) equipped with a nano-electrospray source (Applied Biosystems, Foster City, CA, USA) and operated with the Analyst QS v1.1 data acquisition software. Tandem mass spectra were extracted by ABI Analyst version 1.1. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.0.01). Mascot was searched with a fragment ion mass tolerance of 0.30 Da and a parent ion tolerance of 0.30 Da. Iodoacetamide derivative of cysteine is specified in Mascot as a fixed modification. Deamidation of asparagine and glutamine and oxidation of methionine were specified in Mascot as variable modifications.

Results and Discussion

As with xylem sap of other crops, the xylem sap of *Vitis* species contains low concentration of protein which makes studying xylem sap proteins challenging. In addition, the presence of various compounds such as phenolics, organic acids, amino acids, sugars, and other metabolites makes analysis and characterization of grape xylem sap proteins tedious and difficult. Xylem sap was collected in spring, during which the incidence, multiplication, and movement of *X. fastidiosa* has been reported to be high [22]. Sap collection during morning hours yielded sufficient amount for use in this study. The cut stem was thoroughly rinsed with distilled water before collecting the sap. It has been demonstrated that washing cut stem with water prior to xylem sap collection prevents possible contamination of phloem sap and other adjacent cellular compartments in the sample [15]. Sap was collected from the PD-infected as well as from the control (healthy) plants grown in a screen house (disease-free environment). We constantly monitored PD incidence during the study period to insure plant health. One-dimensional electrophoretic analysis of freeze-dried xylem sap from *V. rotundifolia* (muscadine cv. Dixie Red), *Xylella*, and the blank PD3 broth used to grow *Xylella* showed the presence of a few common protein bands between xylem sap and *Xylella*, indicating the presence of the bacterium in sap (Fig. 1). The presence of these proteins in xylem sap may serve as an indicator of plant health and disease status.

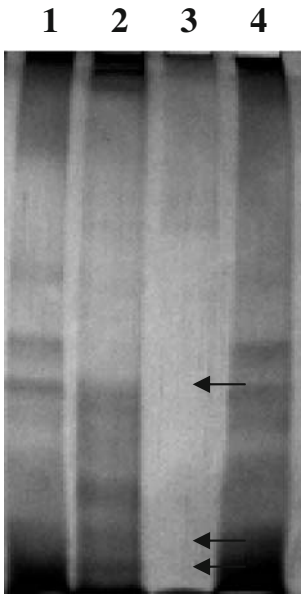

Fig. 1 Similarity in the polypeptide composition of xylem sap of muscadine cv. Dixieland and *Xylella*. *Lanes 1and 4*: xylem sap of Dixieland (Muscadine), *lane 2*: *Xylella*, and *lane 3*: Blank PD3 broth. *Arrows* indicate common polypeptides

Free amino acids and sugar analysis of xylem sap confirmed the existence of a major difference among the *Vitis* species studied. The amount of free amino acids and soluble sugars was higher in PD-susceptible bunch grape (*V. vinifera*) genotypes compared to PD-tolerant muscadine (*V. rotundifolia*) grape genotypes (Figs. 2 and 3). This suggests that the

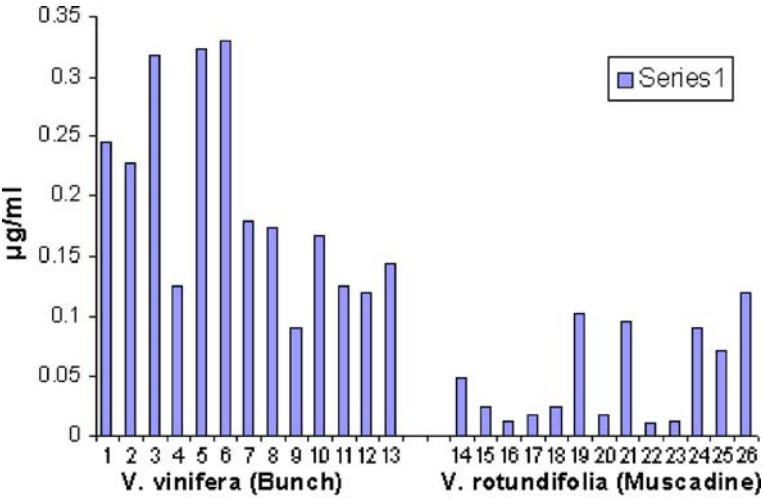

Fig. 2 Variation in the xylem sap free amino acid content in grape cultivars. California Bunch, Lane 1 to 13: Barbera, Ruby Cabernet, Sauvignon Blanc, Chenin Blanc, Fiesta, Napa Gammy, Zinfandel, Petite Sarah, Merlot, Pinot Noir, Fame Seedless, JS2605 and, Chardonnay. Muscadine, Lane 14 to 26: Noble, Alachua, African Queen, Black Beauty, Scuppernong, Pride, Regale, Higgins, Scarlet, Sweet Jenny, Senoia, Carlos and Sterling

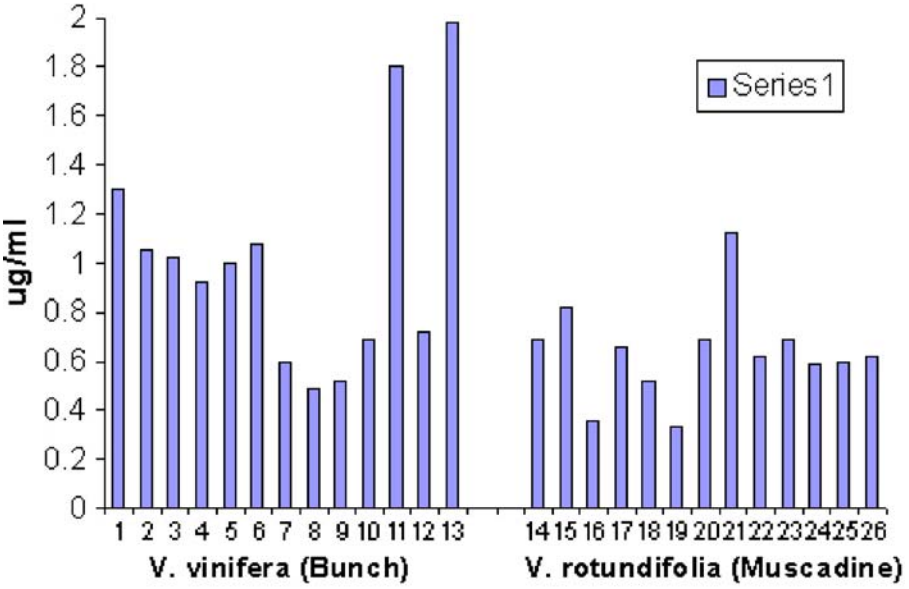

Fig. 3 Variation in the xylem sap sugar content in grape cultivars. California Bunch, Lane 1 to 13: Barbera, Ruby Cabernet, Sauvignon Blanc, Chenin Blanc, Fiesta, Napa Gammy, Zinfandel, Petite Sarah, Merlot, Pinot Noir, Fame Seedless, JS2605 and, Chardonnay. Muscadine, Lane 14 to 26: Noble, Alachua, African Queen, Black Beauty, Scuppernong, Pride, Regale, Higgins, Scarlet, Sweet Jenny, Senoia, Carlos and Sterling

xylem sap of bunch grape are more nutritious than muscadine grapes, which may support *Xylella* growth better than the muscadine. For further differentiation, a high throughput two-dimensional gel electrophoresis coupled with silver staining was used to resolve and detect the xylem sap proteins from PD-tolerant *V. rotundifolia* (cv. Dixieland, Cowart, and Sterling) and Florida hybrid bunch (Lake Emerald, Blue Lake, and Orlando Seedless), and PD-susceptible *V. vinifera* (cv. Fiesta, Petite Sarah, and Pinot Noir) genotypes. The *V. vinifera* cultivars were grown under protective environment to prevent infection and were used as "healthy" controls for comparison with muscadine as they are highly susceptible to PD. The incidence of PD and the activity of the glassy-wing sharpshooter (the vector that carries *Xylella*) are known to be high in southeastern USA. Two-dimensional gel electrophoretic analysis of xylem sap collected on different days during the spring season yielded consistent reproducible polypeptide spots, but their expression levels varied. Since xylem sap protein profiles of grape cultivars within each species were found to be similar, only one profile representing muscadine (cv. Dixieland), Florida hybrid bunch (cv. Lake Emerald), and bunch (cv. Fiesta) are discussed in this report to avoid duplication.

The 2-D PAGE system separated xylem sap proteins into more than 100 polypeptides with p*I*s between 3.5 to 9.0 and molecular weight of (M_r) 20 to 75 kDa (Fig. 4). The majority of the xylem sap proteins appear to be of high (>40 kDa) molecular weight, and they resolved into clusters of multiple spots. The apparent M_r of proteins in each cluster was similar but their p*I*s were slightly different resulting in a series of spots. Most of these clusters focused between pH 4.0 and 7.0 and had M_r of >40 kDa. Such polypeptide clusters have been observed on 2-D PAGE of several seed, leaf, and microbial protein extracts and appear to be a normal occurrence in plant tissue [23–25]. But, the polypeptide profiles of xylem sap within the clusters of different *Vitis* species were found to be distinct and unique (Fig. 4). Polypeptides unique to each genotype were identified by comparing their 2-D

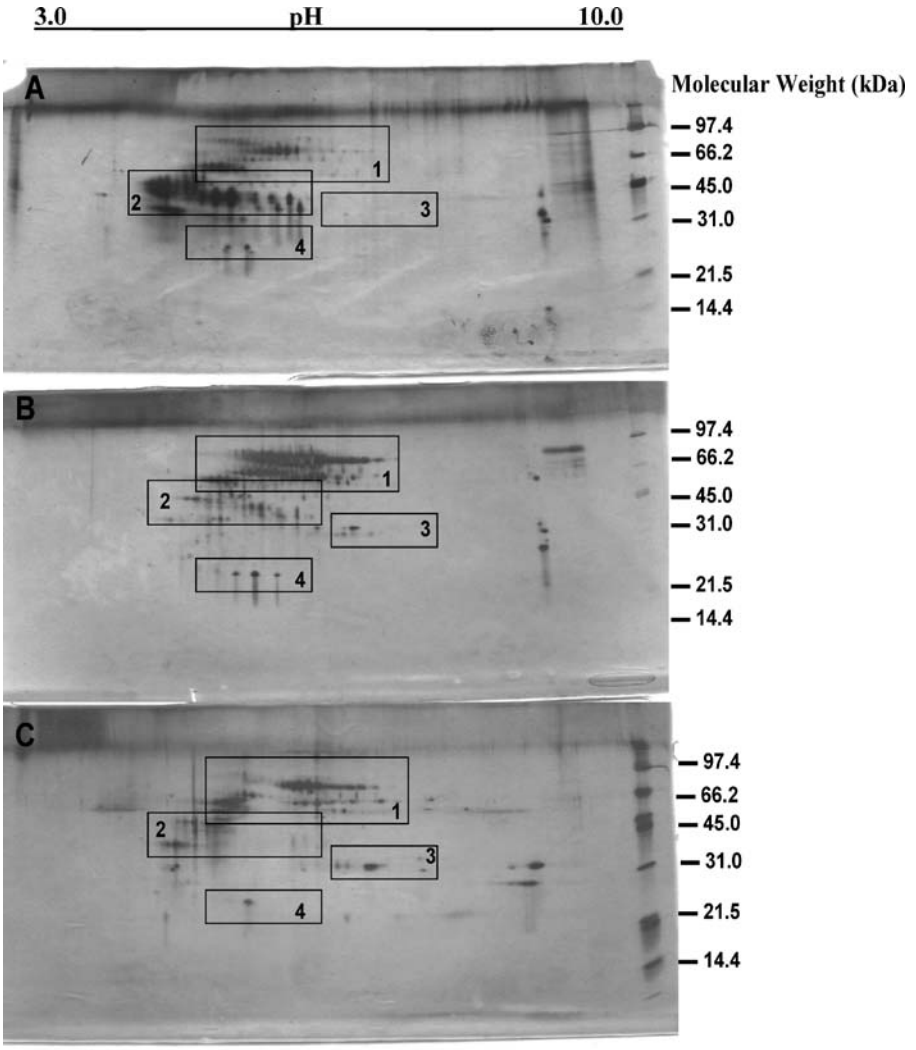

Fig. 4 Polypeptide profile of xylem sap proteins. **a** Dixieland (*V. rotundifolia*, muscadine grape), **b** Lake Emerald (*Vitis* spp., Florida bunch hybrid), **c** Fiesta (*V. vinifera*, bunch grape). Molecular weight standards: 97.4 kDa = phosphorylase b, 66.2 kDa = bovine serum albumin, 45.0 kDa = ovalbumin, 31.0 kDa = carbonic anhydrase, 21.5 kDa = soybean trypsin inhibitor, and 14.4 kDa = lysozyme. The gel regions exhibiting variation in the polypeptide composition are shown in *boxes 1*, *2*, *3*, and *4*

PAGE profiles with each other. Comparison of 2-D gels was carried out both manually and by using PD Quest software (PD Quest 2-D Analysis Software, BioRad Laboratories, Inc.).

This comparison enabled us to identify four regions of the gel with distinct polypeptide composition. A close up view of these regions (box 1 through box 4) showing major differences among the different *Vitis* species is shown in Fig. 5. The three *Vitis* species showed major differences in the polypeptides with M_r range between 55 and 75 kDa and p*I* between 5.0 and 7.0 [Fig. 5—1 (box 1 in Fig. 4)]. As pointed out earlier, these polypeptides resolved as clusters and their number and quantity varied among the *Vitis* species. For ease of comparison, these polypeptide clusters are identified as cluster A, cluster B, cluster C,

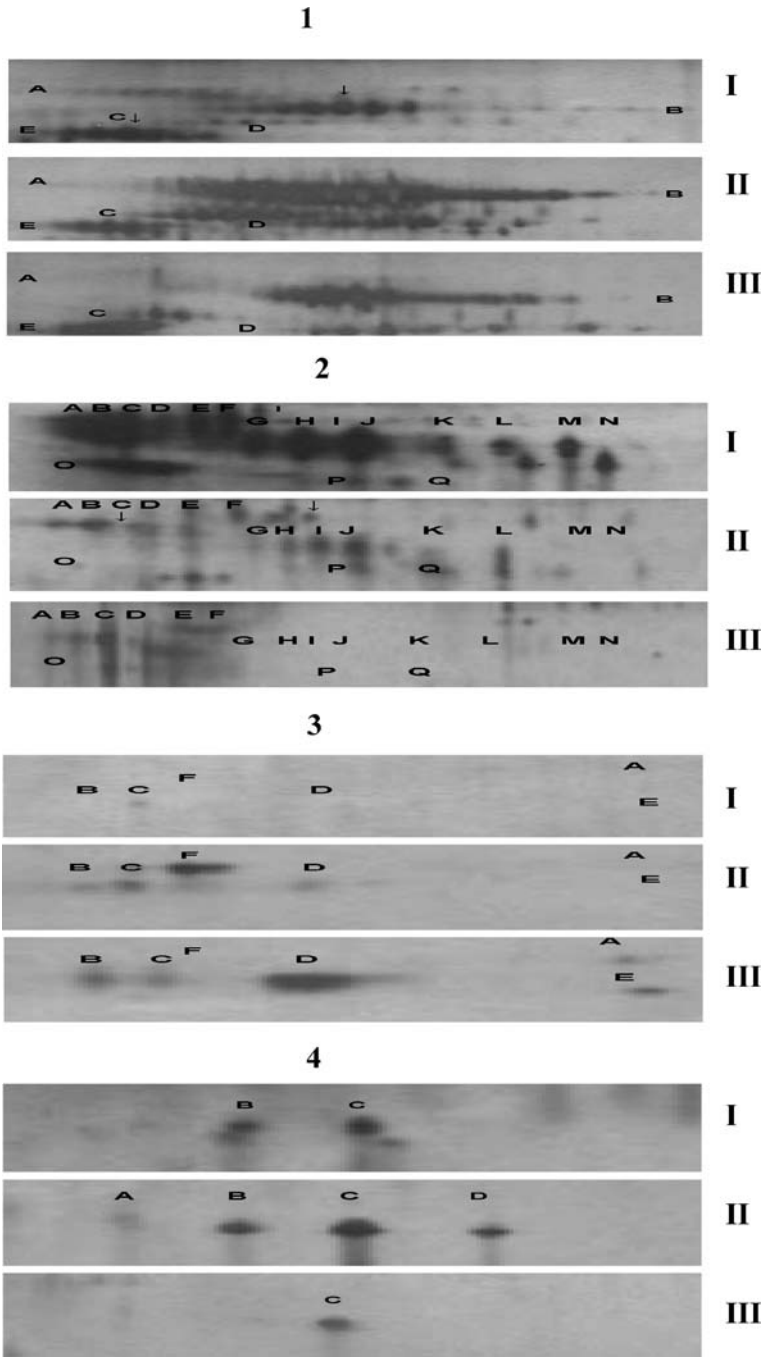

Fig. 5 Enlarged view of *box 1*, *box 2*, *box 3*, and *box 4* from Fig. 1. In *box 1*, polypeptide clusters are identified as clusters A, B, C, D, and E. *I* Dixieland (Muscadine), *II* Lake Emerald (Florida hybrid bunch), *III* Fiesta (bunch)

cluster D, and cluster E based on their mobility (M_r). In the muscadine grape cultivar, all the polypeptides of cluster B and cluster E were present while the polypeptides of cluster D were absent. In the Florida hybrid bunch grape cultivar, polypeptides of all the five clusters (A through E) were present in higher amounts, and in bunch grape cultivar, polypeptides of cluster B, cluster C, cluster D, and cluster E were present but the cluster A proteins were absent. A comparison of the *V. rotundifolia* and *V. vinifera* cultivars showed that the latter lacked cluster A polypeptides while *V. rotundifolia* lacked cluster D polypeptides. Likewise, a comparison of the muscadine and the Florida hybrid bunch grape cultivars showed that both of them contained cluster A, cluster B, cluster C, and cluster E polypeptides but the muscadine grape cultivar lacked the cluster D polypeptides.

Figure 5—2 shows the gel region containing proteins with p*I*s between 4.1 and 6.5 and apparent M_r around 35 to 55 kDa (box 2 in Fig. 1). This region is considered important since it contains the polypeptides unique to the PD-tolerant muscadine and Florida hybrid bunch grape cultivars. Interestingly, these polypeptides were completely absent in the PD-susceptible *V. vinifera* cultivars. As seen in Fig. 5—2, the muscadine grape (cv. Dixieland) contained more than 14 polypeptides (A through N) in this region, while the Florida hybrid bunch grape (cv. Lake Emerald) contained polypeptides A, B, D, E, G, H, I, J, and L at reduced levels and lacked polypeptides C, F, K, M, and N. In the bunch grape cultivar (*V. vinifera* cv. Fiesta), polypeptides G, H, I, J, K, L, M, and N were completely absent while the other polypeptides were present at significantly reduced levels. These data show that the expression of polypeptides G, H, I, J, K, L, M, and N was high in muscadine [cv. Dixieland (PD-tolerant)], low in Florida hybrid bunch grape [cv. Lake Emerald (PD-tolerant)], and absent in bunch grape [cv. Fiesta (PD-susceptible)]. In addition, Fig. 5—3 showing gel region (box 3 in Fig. 4) also showed differences in polypeptide composition among the *Vitis* species. In this region, the bunch grape (cv. Fiesta) contained five polypeptides (A through E) but lacked polypeptide F, while the Florida hybrid bunch (cv. Lake Emerald) contained reduced levels of polypeptides B, C, and D and a unique polypeptide F but lacked polypeptides A and E. None of these polypeptides (A through F) were found in *V. rotundifolia* (cv. Dixieland). Furthermore, the polypeptides identified in box 4 (Fig. 4) also varied among the *Vitis* species. Florida hybrid bunch grape contained four polypeptides (A, B, C, and D) in this region, muscadine grape had two (B and C), and bunch grape had only one (C) polypeptide. These data further suggest that although xylem sap of *Vitis* species contains certain common proteins, differences occur in the expression of certain unique polypeptides.

The polypeptide data including their p*I* and M_r found in different *Vitis* species are summarized in Table 1. The polypeptides present in all the three species are termed as "common" while the polypeptides that differed among the species are termed "variable". Of more than 100 polypeptides observed in the xylem sap 2-D PAGE profiles of *Vitis* species, only few polypeptides were found to be common to all the three species. The remaining polypeptides were either absent or were present in variable amounts among the *Vitis* species indicating the existence of wide genetic differences in xylem sap protein composition.

Based on comparisons of *Vitis* species xylem sap 2-D PAGE profiles, ten polypeptides showing major differences were selected for sequencing by LC/MS/MS. Partial amino acid sequencing data showed that polypeptide spots L and P (Fig. 5—2) were found to be similar to β-1, 3-glucanase and peroxidase, both of which are known to be involved in plant defense (Table 2), whereas spots G, H, and J (Fig. 5—2) were found to be similar to oxygen-evolving enhancer protein (OEE1), which is involved in generating oxygen. The occurrence of similar or identical gene products slightly differing in their p*I* and M_r is a common feature observed in 2-D analysis and can be attributed to post-translational modifications of distinct amino acids of one single gene product. Protein sequence searches

Table 1 Characteristics of xylem sap polypeptides from different *Vitis* species.

Polypeptide			*Vitis* species		
Group	p*I* (pH)	M_r (kDa)	*V. rotundifolia*	*Vitis* spp.	*V. vinifera*
Common polypeptides					
Box 1					
B	5.0–6.5	66	+	+	+
C	5.0–6.5	66	+	+	+
E	4.5–5.0	50	+	+	+
Box 2					
A	4.2	45	+	+	+
B	4.4	45	+	+	+
D	4.5	45	+	+	+
E	4.6	45	+	+	+
F	4.8	45	+	+	+
Box 3					
C	6.3	31	+	+	+
Box 4					
C	5.6	23	+	+	+
Variable polypeptides					
Box 1					
A	4.5–5.0	70	+	−	−
D	6.0–7.0	50	+	−	+
Box 2					
C	4.3	45	+	−	−
G	5.0	31	+	+	−
H	5.2	31	+	+	−
I	5.3	31	+	+	−
J	5.5	31	+	+	−
K	5.8	25	+	−	−
L	6.0	25	+	+	−
M	6.2	25	+	+	−
N	6.3	25	+	−	−
Box 3					
A	7.0	35	−	−	+
B	6.2	31	−	+	+
D	6.5	31	−	+	+
E	7.2	28	−	−	+
F	6.5	45	−	+	−
Box 4					
A	5.2	23	−	+	−
B	5.4	23	+	+	−
D	6.0	23	−	+	−

+ present, − absent, M_r molecular weight, p*I* isoelectric point

Table 2 Partial sequences of xylem sap proteins from *V. rotundifolia* obtained by mass spectrometric analysis that showed high similarity (95% identity) to database entries.

Spot no.[a]	Sequence	Mascot score	No. of unique peptides	GI accession No.	Protein similar to	Organism matched	UniProtKB accession no.
L	NIFNAISAAGLGNQIK	88.2	2	CAB91554	β-1, 3-glucunase	*V. vinifera*	P86102
	VSTAIDTGVLGTSYPPSK	76.4					
P	DNTAKEKDSPANLSLR	66.2	4	18418208	Peroxidase	*A. thaliana*	P86103
	QAGVLFSDQSLFTSAR	90.1					
	NTFDNAYYIALQR	55.2					
	TCAAGDNAEQPLDPSR	42.4					
G	TNAENEFVTI KK	54.1	1	P84718	OEE1	*P. strobus*	P86104
H	TNAENEFVTI KK	52.2	1	P84718	OEE1	*P. strobus*	P86104
J	TNAENEFVTI KK	50.6	1	P84718	OEE1	*P. strobus*	P86104

[a] The spots were excised from box 2 of Fig. 1

of the other five protein spots failed to show any similar matches with the known database and, hence, are identified as proteins of unknown function (data not shown).

The beta-1, 3-glucanases (spot # L), peroxidase enzyme (spot # P), and oxygen-evolving enhancer protein (spots # G, H, and J) were expressed only in PD-tolerant *Vitis* species (muscadine and Florida hybrid bunch grape), which may be partly responsible for their PD tolerance. Furthermore, high expression of these proteins by muscadine genotypes compared to other *Vitis* species may be responsible for their superior PD-tolerance characteristics than the hybrids and bunch.

Beta-1, 3-glucanases are a class of proteins that are often associated with pathogen-related proteins and are believed to mediate defense responses upon pathogen infection [26]. The occurrence of β-1, 3-glucanase proteins has been reported only in tomato xylem sap [7, 11]. Interestingly, glucanases have been suggested to act in a synergistic manner with thaumatin-like proteins that can bind to β-1, 3-glucans [27] and have not been reported to occur in xylem sap of healthy, unchallenged plants, but were found only after infection [11]. Although the plants we studied were not actively challenged with *Xylella*, the expression of β-1, 3-glucanase protein indicates that the field-grown PD-tolerant *Vitis* species were naturally infected with *Xylella*.

Another class of pathogen-related proteins induced upon *Xylella* infection in PD-tolerant *Vitis* species is peroxidase (spot # P), which is an oxidative enzyme [28]. This class III secretary peroxidase is known to perform versatile functions along with regulation of H_2O_2 level and oxidation of toxic compounds upon pathogen infection [29]. The induction of a cationic peroxidase in xylem vessels has been reported in an incompatible interaction between the vascular pathogen *Xanthomonas oryzae* pv *oryzae* and rice [30]. In the compatible response, the antibodies did not detect the presence of peroxidase until 48 h after infection. This suggests that the expression of peroxidase in xylem sap is due to *Xylella* infection. Expression of xylem sap peroxidase has also been reported in apple, peach, and pear and has been suggested to play a role in plugging damaged vascular tissue [7]. The expression of β-1, 3-glucanase and peroxidase in PD-tolerant *Vitis* species indicates that the defense pathways, jasmonic and salicylic acid pathways, were induced upon *Xylella* infection. The accumulation of pathogen-related proteins suggests that the plant has acquired systemic resistance against a broad spectrum of microorganisms, which require salicylic acid and confer long-lasing protection [31, 32].

Interestingly, we also found the expression of three unique polypeptides (spots # G, H, and J), which were found identical to OEE1. OEE1 has been suggested to bind with calcium ions, which have been shown to be essential for maximizing the rates of oxygen evolution during photosystem complex II [33]. Further, full-length amino acid sequencing of this unique polypeptide may reveal the differences in the amino acid composition present compared to OEE1. *X. fastidiosa* lacks cytochrome with high oxygen affinity. Instead, it possesses simple and quite unusual aerobic respiratory complex [34]. In silico functional analysis of *Xylella* has shown the presence of the least energy-efficient type of aerobic respiration of any known organism reported to date [34]. They also suggested that *Xylella* clearly prefers anerobic respiration based on sulfur metabolism. Dalke [35] reported that *Xylella* can breathe with or without oxygen, but both energy pathways are extremely inefficient. This can be utilized as a potential target for disease control. Expression of OEE1-type polypeptide in xylem sap of PD-tolerant *Vitis* species suggests that it may be involved in generating higher levels of oxygen in sap, hampering normal respiratory process of *Xylella* and thus affecting its growth. This result suggests that the expression of beta-1, 3-glucanases, peroxidase enzyme, and oxygen-evolving enhancer protein along with few unknown proteins by the PD-tolerant *Vitis* species is the vital reason for their tolerance to PD. Further, it would be interesting to investigate how these proteins affect the survival of *X. fastidiosa*, and how *X. fastidiosa* overcomes the toxic effect of these proteins and what role if any these proteins play in PD tolerance of *V. rotundifolia*.

Conclusion

The PD tolerance level of *Vitis* species was found to vary depending upon their xylem sap composition. Our results revealed the existence of major differences in xylem sap free amino acids, soluble sugars content, and protein composition among *Vitis* species. The higher free amino acids and soluble sugars content in *V. vinifera* clearly suggests *Xylella* preference to this species perhaps because of its suitability for *Xylella* growth. Expression of unique proteins by PD-tolerant muscadine and Florida hybrid bunch grape genotypes upon infection indicates their defense mechanism against *Xylella*. The function of most of these proteins is unknown except for five proteins (β-1, 3-glucanase, peroxidase, and OEE1) which are involved in plant defense and oxygen enhancement. This clearly suggests that *V. rotundifolia* and Florida hybrid bunch grape genotypes were able to express these proteins following infection, while *V. vinifera* lacks this capability which makes them susceptible to PD. In addition, it was shown that xylem sap of the cultivar Noble (*V. rotundifolia*) was more resistant to protein breakdown by *X. fastidiosa* compared to that of cultivar Chardonnay (*V. vinifera*) [36], suggesting that *V. rotundifolia* xylem sap may possess protease inhibitor activity. Further study of these uniquely expressed polypeptides should help determine their role in PD tolerance.

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