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e-Xtra*

Proteomic Comparison of Needles from Blister Rust-Resistant and Susceptible *Pinus strobus* Seedlings Reveals UpRegulation of Putative Disease Resistance Proteins

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In order to characterize a hypersensitive-like reaction in selected *Pinus strobus* seedlings to *Cronartium ribicola*, a proteomic comparison of needles from resistant and susceptible seedlings was undertaken using two-dimensional gel electrophoresis (2-DE). The results revealed 19 polypeptides specific to resistant seedlings and seven of these specific to infected resistant seedlings. There were 13 polypeptides up-regulated (≥3-fold increase) in resistant family P327 in comparison to needle tissue from susceptible and mock-inoculated seedlings. Electrospray ionization liquid chromatography and tandem mass spectrometry was used to sequence 11 proteins from the 2-DE gels. Sequences obtained from electrospray ionization liquid chromatography and tandem mass spectrometry were used for MS-BLAST and Pro-ID database searches allowing identification with a 95 to 99% confidence level. Six proteins were determined to be homologs of proteins with known roles in disease resistance, five were determined to be homologs of members of the leucine-rich repeat (LRR) superfamily, and one was a homolog of heat shock protein 90, a protein that serves as a cofactor for certain LRR proteins. This is the first report of members of the LRR family with functional homologs in *Pinus strobus* and of a molecular basis for white pine blister rust resistance in *Pinus strobus*.

Additional keywords: LRR disease resistance.

White pine blister rust (WPBR) caused by *Cronartium ribicola* is the most damaging disease of five-needled pines (white pines) in North America (Kinloch and Littlefield 1977; Patton 1967). The pathogen was introduced to both coasts of North America on infected nursery stock from Europe a century ago and has caused widespread damage to white pine populations throughout North America (Kinloch and Littlefield 1977; Jurgens et al. 2003). Because North American white pines did not co-evolve with the pathogen, little resistance has been observed in natural populations (Kinloch and Dupper 2002; Patton 1972).

Resistant individuals of *Pinus lambertiana*, *Pinus monticola* and *Pinus strobus*, have been observed (at very low frequencies) and have been the focus of investigations to develop resistance breeding programs (Jurgens et al. 2003; Kinloch and Dupper 2002; Riker et al. 1953). Candidate genes for resistance have been putatively identified at low frequencies (designated *Cr1* and *Cr2*, respectively) in western species (*Pinus lambertiana* and *Pinus monticola*) and employed in both species (Kinloch and Dupper 2002). The phenotype conditioned by these alleles is a classic hypersensitive response similar to other *R* gene–mediated hypersensitive responses (HR) in a number of other plant species (Kinloch and Littlefield 1977; Liu and Ekramoddoullah 2003). Although these resistance genes have not been cloned, recent work by Liu and Ekramoddoullah (2003) identified resistance gene analogs (RGA) from these species using degenerate polymerase chain reaction (PCR).

Proteomic analyses have been very limited in the WPBR pathosystem and have been restricted to western species of white pine (Ekramoddoullah and Hunt 1993; Ekramoddoullah and Taylor 1996; Davidson and Ekramoddoullah 1997). In these studies, two up-regulated proteins (or genes) were identified in needles of resistant seedlings, and another protein that was found to be up-regulated during the autumn, a period associated with cold-hardiness, was identified and determined to be homologous to a heat-shock protein (Ekramoddoullah and Hunt 1993).

The molecular basis of resistance in *Pinus strobus* has been sparingly studied. Jurgens and associates (2003) provided a synopsis of histological studies of host reactions of needle tissue to infection by *C. ribicola*. Seedlings derived from parent clone P327 (Patton and Riker 1966) demonstrated a hypersensitive reaction. Infected seedlings showed an arrest in fungal growth and mesophyll cells collapsing in tissue surrounding the infection site at 7 weeks after infection, providing evidence for HR-like resistance. This confirmed phenotypic observations that this family developed smaller spots after infection that do not develop into systemic infection as frequently as in seedlings from susceptible families (Jurgens et al. 2003).

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*The *e*-Xtra logo stands for "electronic extra" and indicates that Figure 1, which appears in black and white in print, appears in color on-line.

This study used proteomic comparisons between resistant and susceptible families to investigate plant defenses against *C. ribicola* in *Pinus strobus.* Proteins up-regulated in the HR-like resistance phenotype in controlled-cross progeny of the resistant selection P327 were identified and classified and will be used in future studies to investigate their function in WPBR resistance. It is the ultimate goal of this study to provide a mechanism that will allow improved treatment and breeding to reduce the impact of WPBR in *Pinus strobus* populations.

RESULTS

Seedling inoculations.

Evidence of WPBR was phenotypically visible 3 weeks postinoculation (wpi), as indicated by characteristic spot development on primary needles of inoculated seedlings. The WPBR spots had developed yellow discoloration and were more pronounced after 4 weeks; spots were also smaller on resistant seedlings at this time. No symptoms developed on mock-inoculated seedlings of either family after eight months. Histological sections (Fig. 1) revealed that colonization by the fungal hyphae were restricted at 4 weeks in needles of the resistant P327 seedlings, whereas the susceptible H111 seedlings had more extensive colonization.

Molecular weight and isoelectric point (pI) measurements.

Samples obtained from needles contained 8.19 to 9.07 µg of total foliar protein per microliter of total foliar proteins extracted from the samples. Approximately 1,000 protein spots (Fig. 2) with a molecular mass range of 14 to 200 kDa and pI range of 4.2 to 7.7 were resolved per get.

Computational gel comparisons.

Computational gel analyses revealed differences in expression of proteins among samples and treatments (Table 1, Figs. 2 through 4). Nine polypeptides were found exclusively in H111 (inoculated and control) and six were found only in P327. Comparison of mock-inoculated samples revealed a single polypeptide from H111 control needles (spot 939). Comparison of inoculated samples revealed seven spots exclusively in P327 inoculated and two that were exclusive to H111 inoculated. Differential expression comparisons of inoculated and mock-inoculated showed five polypeptides that were up-regulated (≥3-fold increases) in H111 control versus H111 inoculated seedlings; conversely, there were 31 polypeptides that were up-regulated in H111 inoculated versus H111 control seedlings. In P327, six polypeptides were up-regulated in P327 control versus P327 inoculated seedlings and 13 polypeptides were up-regulated in P327 inoculated versus P327 control. Comparison between inoculated seedlings showed six polypeptides were up-regulated in H111 inoculated versus P327 inoculated seedlings and 23 polypeptides were up-regulated in P327 versus H111 inoculated seedlings.

Electrospray spectral analysis.

Of the initial 19 spots that were excised from the gels, 11 provided adequate full-scan mass spectra to submit for mass spectrometry tandem mass spectrometry (MS/MS) and subsequential (Figs. 5 and 6) de novo sequencing and data base searches. Peptides that represented trypsin and keratin were excluded prior to database searching. Two spots (spots 941 and 948) failed to match any protein using either MS-BLAST or Pro-ID. Five of the nine identified proteins had homology to known disease-resistance proteins. Spot 502, which migrated very close to the tropomyosin control spot (Fig. 4), matched tropomyosin, suggesting a contamination was present. Eight peptides from spot 732 matched a chloroplast latex aldolase-like protein from *Manihot esculenta* (AY818399). Two peptides from spot 732 matched an ATP-dependent protease from *Huperzia lucidula* chloroplast (AY660566). Spot 785 had three peptides that matched a cytosolic phosphoglycerate kinase from *Nicotiana tabacum* (Q42961). There were two peptides from spot 959 that matched cytochrome c oxidase subunit II from *Polytomella* sp. strain 'Pringsheim 198.80'(Q9AQY5). Polypeptide spot 48 had six peptides that matched (total score 395) a putative heat shock protein (At2g04030) from *Arabidopsis thaliana* (AY094422) that has high similarity to the well-characterized heat-shock protein 90b(HSP90). Two additional peptides from spot 48 matched another putative HSP, HSP gp96 from *Strongylocentrotus purpuratus* (Q868Z7), and two other peptides from spot 48 matched HSP htpG from *Helicobacter pylori* (Q9ZMM2). It is likely that spot 48 represents a HSP that is expressed in *Pinus strobus* in response to infection by *C ribicola.* Spot 533 had 10 peptides that matched an oxygen-evolving enhancer protein from *Lycopersicon esculentum* (P23322) and two peptides that matched a putative glyoxalase from *Oryza sativa* (Q75GB0). Four other peptides from spot 533 had high similarity to a NAD(P)-dependent glyceraldehyde-3-phosphate dehydrogenase from *Fischerella*

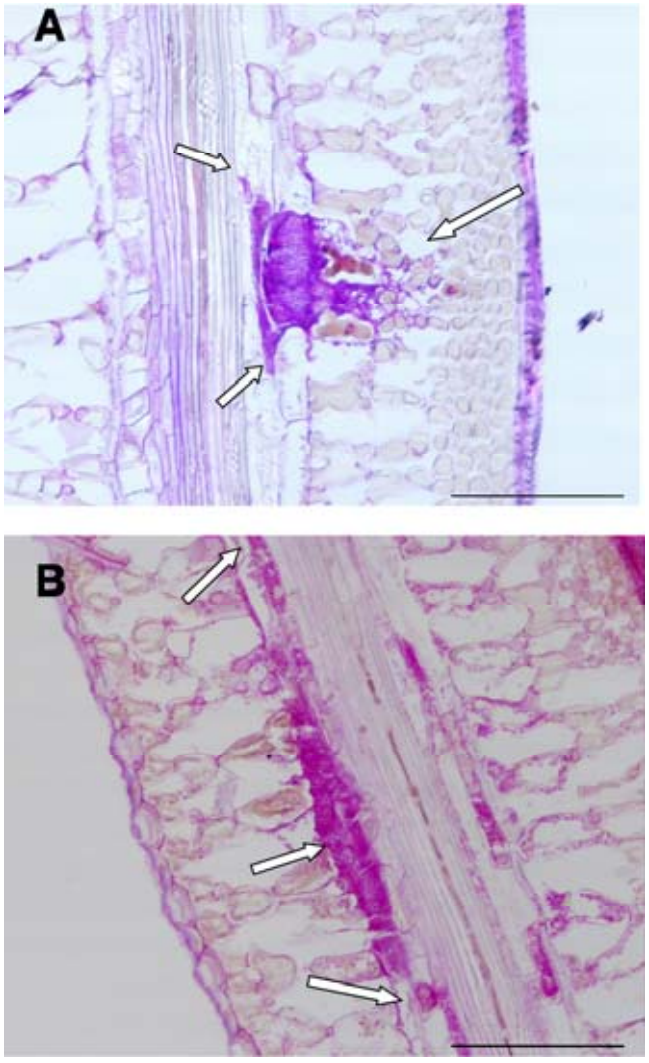

Fig. 1. Histological sections stained with Schiff's reagent of **A,** resistant and **B,** susceptible needles at 4 weeks postinoculation (100× magnification). The hyphae of *Cronartium ribicola* are restricted in A and are not penetrating vascular tissue. Whereas in B, the hyphae are not restricted and have colonized the vascular bundle. Arrows indicate extent of hyphal colonization. Bar = approximately 50 µm.

sp. strain UTEX 1829 (Q8VNK8) and one peptide matched a putative leucine-rich repeat (LRR) disease-resistance protein/transmembrane receptor kinase—At2g01210 from *Arabidopsis thaliana* (NP_178230). Spot 765 had two peptides each matching the following proteins: disease-resistance protein (Toll interleukin-1 receptor-like domain-nucleotide binding site [TIR-NBS]-LRR) from *Arabidopsis thaliana* (Q94AG2), H+-ATPase I subunit from *Pinus koraiensis* (Q85WS7), NADH dehydrogenase subunit from *Phymaturus punae* (Q71SE3), and ATP synthase B chain from *Pinus thunbergii* (O62939). Spot 765 had one peptide that had high similarity to photosystem II oxygen-evolving complex protein 2 from *Pinus monticola* (Q7M1Y8). There were five peptides from spot 770 that matched a 2-Cys peroxiredoxin from *Pisum sativum* (Q93X25). Spot 770 had seven peptides that matched an ATP-dependent protease subunit from *Pinus koraiensis* (Q85X43) and a LRR protein from *Trypanosoma brucei* (Q7YW17). One peptide from spot 963 matched acid phosphatase from *Archaeoglobus fulgidus* (O29320) and one peptide matched a putative LRR disease-resistance protein/transmembrane receptor kinase (At2g01210) from *Arabidopsis thaliana* (NP_178230).

DISCUSSION

To understand and provide a possible mechanism for resistance to WPBR, this study begins to evaluate and characterize proteins expressed during *C. ribicola* infection of a resistant *Pinus strobus* family. This is the first proteomic study of *Pinus strobus* during infection by *C. ribicola* and the first report of LRR homologs in *Pinus strobus*.

The differences between susceptible and resistant protein profiles were substantial and provide clear evidence for genotypic differences between the seedlings, which are responsible for the phenotypic disease traits observed. Although the specific roles of these proteins remain unknown, the two-dimensional electrophoresis (2-DE) experiments reported here provide strong

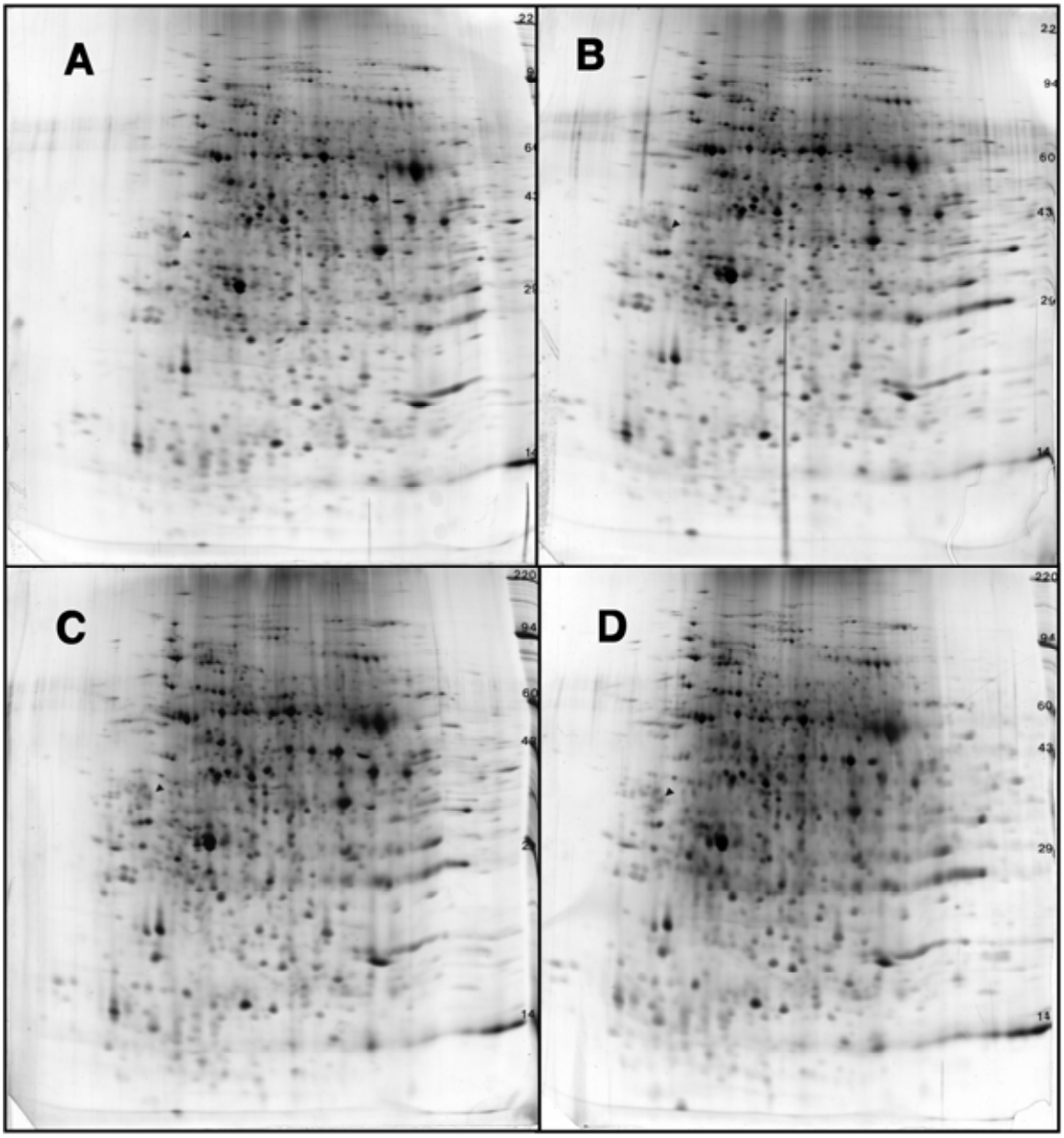

Fig. 2. Silver-stained two-dimensional polyacrylamide electrophoresis gels showing separation of total proteins from needles of **A,** P327 × P327 mock-inoculated, **B,** P327 × P327 inoculated, **C,** H111 × H111 mock-inoculated, and **D,** H111 × H111 inoculated. Arrows indicate locations of spot corresponding to tropomyosin control.

evidence for involvement in the host-pathogen interaction. In this study, we chose highly up-regulated (>3-fold) proteins for further study; however, there were numerous peptides that were slightly up- or down-regulated that may also be important for understanding how resistant and susceptible selections differ in response to pathogen attack.

The use of the MS-BLAST protocol for identifying protein sequences from unsequenced organisms was required to enable the use of proteomic strategies previously restricted to organisms with published genome sequences (Dreger 2003; Shevchenko et al. 2001). This protocol provided a successful identification rate of 80%, demonstrating its efficacy in homology searches. Those two proteins that were unidentifiable produced weak MS chromatogram signals and is likely to be the primary reason for the poor sequence quality. Although this method does not ensure that all proteins evaluated were from *Pinus strobus* (rather than from endophytes or *C. ribicola*), using proteins that differed in regulation between the resistant and susceptible and infected and mock-inoculated seedlings makes it more likely that they are plant proteins. In addition, several of the disease-resistance proteins identified matched homologs in other plant species, including pine.

The identification of the regulation of heat shock-like protein is significant for several reasons. HSP have been implicated in disease resistance in several studies (Dangl and Jones 2001; Hubert et al. 2003; Liu et al. 2004; Lu et al. 2003). HSP90, a HSP with homologs in several plant species, has been identified as an important component of the HR response (Dangl and Jones 2001; Takahashi et al. 2003). In *Arabidopsis thaliana*, Takahashi and associates (2003) demonstrated that HSP90 functions as a molecular chaperone that was shown by yeast two-hybrid studies to be essential for resistance gene RPS2–mediated HR response to *Pseudomonas syringae* pv. *tomato* DC3000 (*avrRpt2*) and HSP90 (Hubert et al. 2003) has been demonstrated to be crucial for the function of the *Arabidopsis* resistance gene RPM1. HSP90's role in proteasome-mediated degradation of pathogen proteins has also been well-documented (Nimchuck et al. 2003). It has also been recently implicated by virus-induced gene-silencing as an essential component of the nonhost and HR responses in *Nicotiana benthamiana* to infection by *Pseudomonas syringae* (Kanzaki et al. 2003). Gene silencing studies (Lu et al. 2003) have provided strong evidence of the contribution of HSP90 in *Pto* resistance, *Rx*-mediated resistance against *Potato virus X*, and *N*-mediated tobacco mosaic virus resistance. It is interesting to note that, in the current study reported in this paper, both LRR-like resistance proteins similar to many of the *R* genes reported as requiring HSP90 and the HSP90 homolog (spot 48) were up-regulated in the infected P327, a genotype that responds to infection in a HR-like manner. This would make further studies of this protein justified, especially in the WPBR pathosystem.

The NBS-LRR superfamily of resistance genes serves as the dominant group of plant disease-resistance genes (Kobe and Deisenhofer 1994; Martin et al. 2003). This pleiotropic, very large, and functionally diverse group contains many well-known *R* genes (Nimchuk et al. 2003), including some that appear to function in a gene-for-gene manner and others that are apparently involved in quantitative resistance (Hammond-Kosack and Jones 1997). Given the large number of LRR resistance–gene analogs that have been found in dicot species (Dixon et al. 1996; Gachamo et al. 2003) and gymnosperms (Liu and Ekramoddoullah 2003), it would not be surprising to find *R*-gene analogs in *Pinus strobus*. However, it is significant that some of the proteins identified in this study belong to the LRR class of resistance proteins, since most *R*-gene transcripts are found in very low abundance, even after induction in response to a pathogen attack (Jones 2000).

Proteins up-regulated in P327 after inoculation (spot 533 and 963) matched a putative LRR disease-resistance pro-

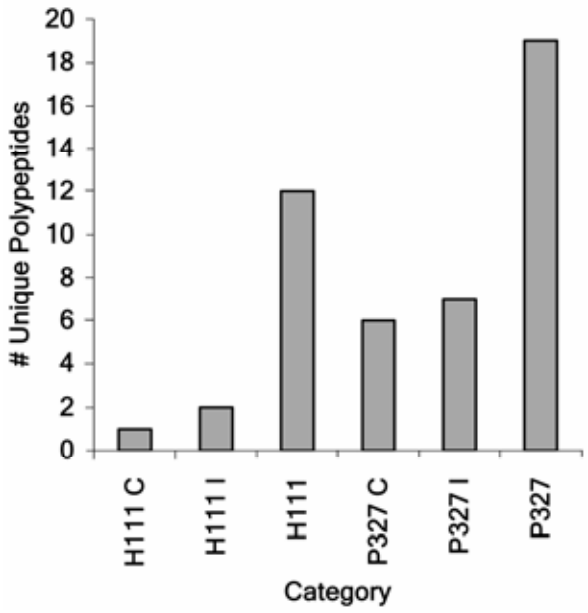

Fig. 3. Polypeptides unique to only one genotype or treatment. H111 only or P327 only indicates that those polypeptides were found in both treatments but in only one genotype.

Table 1. Mean spot intensities (for duplicate two-dimensional gel electrophoresis [2-DE]), isoelectric point (pI), and molecular mass[a]

Spot #[b]	pI[c]	Mol. Mass (kDa)	H111 Control	H111 Inoculated	P327 Control	P327 Inoculated	P327C vs. P327 I[d]	H111 I vs. P327 I
48	5.6	97.6	nd	0.01	0.01	0.05	nc	*–11.27*
502	5.3	37.5	0.01	0.00	0.03	0.03	–1.08	*–8.35*
533	5.7	35.1	0.01	0.02	0.20	0.12	1.62	*–7.47*
732	5.6	25.2	0.03	0.01	0.08	0.07	1.12	*–6.16*
765	7.1	22.1	0.01	0.01	0.01	0.11	*–8.16*	*–7.32*
770	5.7	22.8	0.02	0.02	0.01	0.09	*–11.16*	*–5.53*
785	5.3	22.1	0.10	0.10	0.50	0.35	1.42	*–3.49*
941	nd	nd	nd	nd	0.00	0.00	1.06	…
948	5.1	28.0	nd	nd	0.04	0.03	1.14	…
959	7.4	83.1	nd	nd	nd	0.01	…	nc
963	6.1	81.5	nd	nd	nd	0.02	…	nc

[a] Spots were excised from 2-DE gels for liquid chromatography-tandem mass spectrometry sequencing.
[b] Spot numbers in bold indicate proteins that were determined to be defense-related.
[c] Nd = not determined.
[d] Italics indicate ≥3-fold increases for P327C vs. P327I and H111I vs. P327I. nc = not confirmed on duplicate gels.

tein/transmembrane receptor kinase—At2g01210 from *Arabidopsis thaliana* (NP_178230). This protein is a member of disease-resistance class 5 (Hammond-Kosack and Jones 1997). This class contains non-TIR-NBS-LRR characterized by an extracellular LRR, a membrane-spanning region, and a cytoplasmic serine kinase domain (Hammond-Kosack and Jones 1997). Examples of this class include the *Xa-21* gene from rice, which confers resistance to *Xanthomonas oryzae* pv. *oryzae* (Hammond-Kosack and Jones 1997). Another class of protein up-regulated in response to *C. ribicola* infection in P327 (spot 765) is a member of disease-resistance class 3b (Hammond-Kosack and Jones 1997), a class that contains proteins with a *Drosophila* TIR in addition to the NBS-LRR homology. This class contains the *N* gene from tobacco, *L6* and *M* from flax, and *RPP5* from *Arabidopsis*. The *L6* and *M* genes in flax confer resistance to another obligate biotrophic pathogen, *Melampsora lini.* It will be of interest to determine how it might function in response to *C. ribicola* infection and

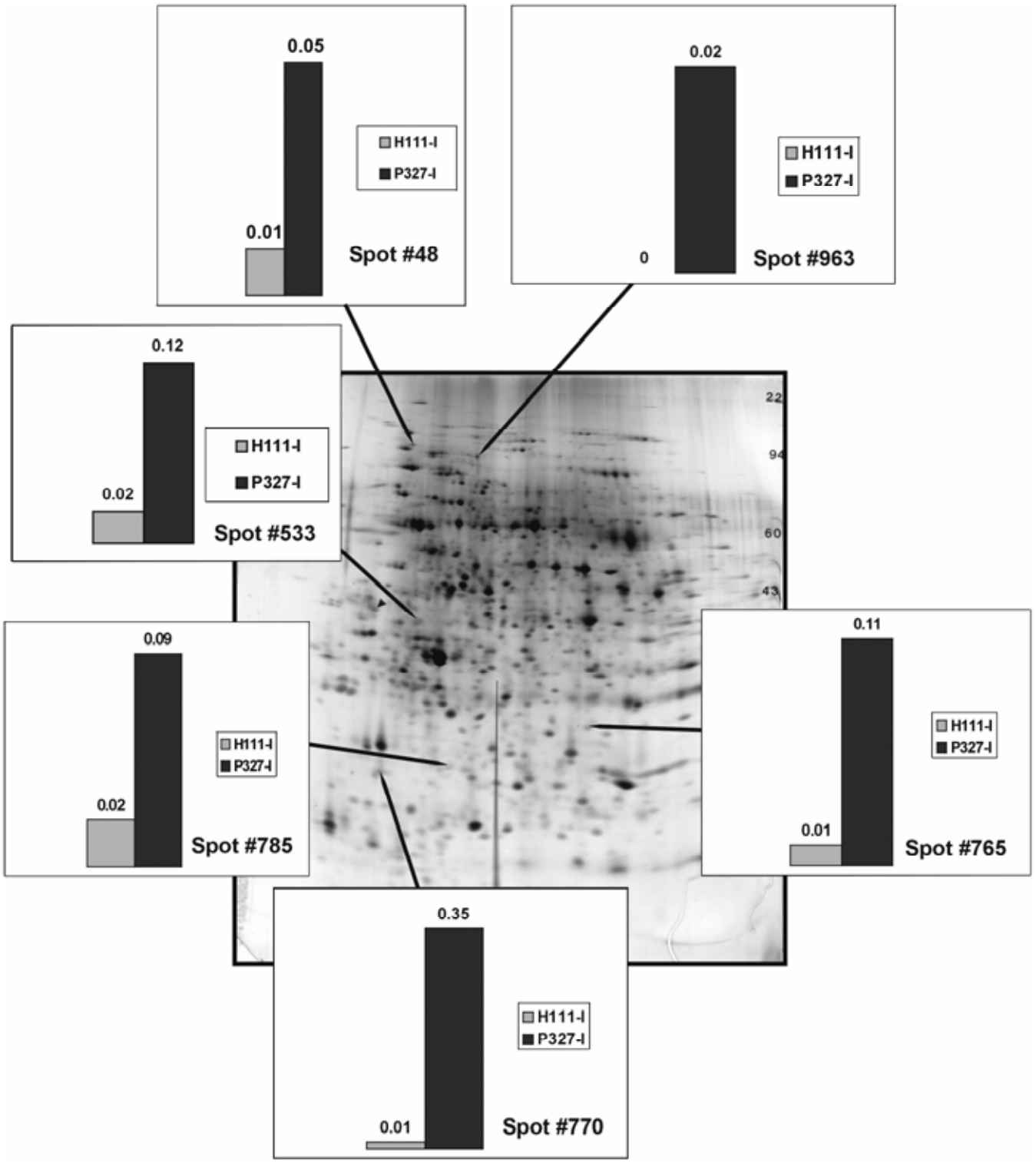

Fig. 4. Two-dimensional gel electrophoresis image of P327-inoculated showing spots that were sequenced and matched putative resistance proteins in database searches. Lines indicate spot locations. The histograms compare the mean difference in spot intensity between H111-inoculated and P327-inoculated.

to determine if it has functional characteristics similar to other rust-resistance genes, such as those in flax (Hammond-Kosack and Jones 1997). Although spot 770 contained an LRR and transmembrane domain and could be classified with class 5, it also contained a peroxiredoxin domain and a protease domain that are members of pathogenesis-related (PR) protein family PR7 (Gachamo et al. 2003). Spot 785 contains a kinase domain that has long been implicated in general disease resistance (Romeis 2003).

This paper provides the first evidence of resistance proteins being up-regulated in *Pinus strobus* in response to *C. ribicola* infection. Liu and Ekramoddoullah (2003) recently reported the presence of TIR-NBS-LRR RGA in *Pinus monticola*—a close relative of *Pinus strobus*. Sequence homology revealed

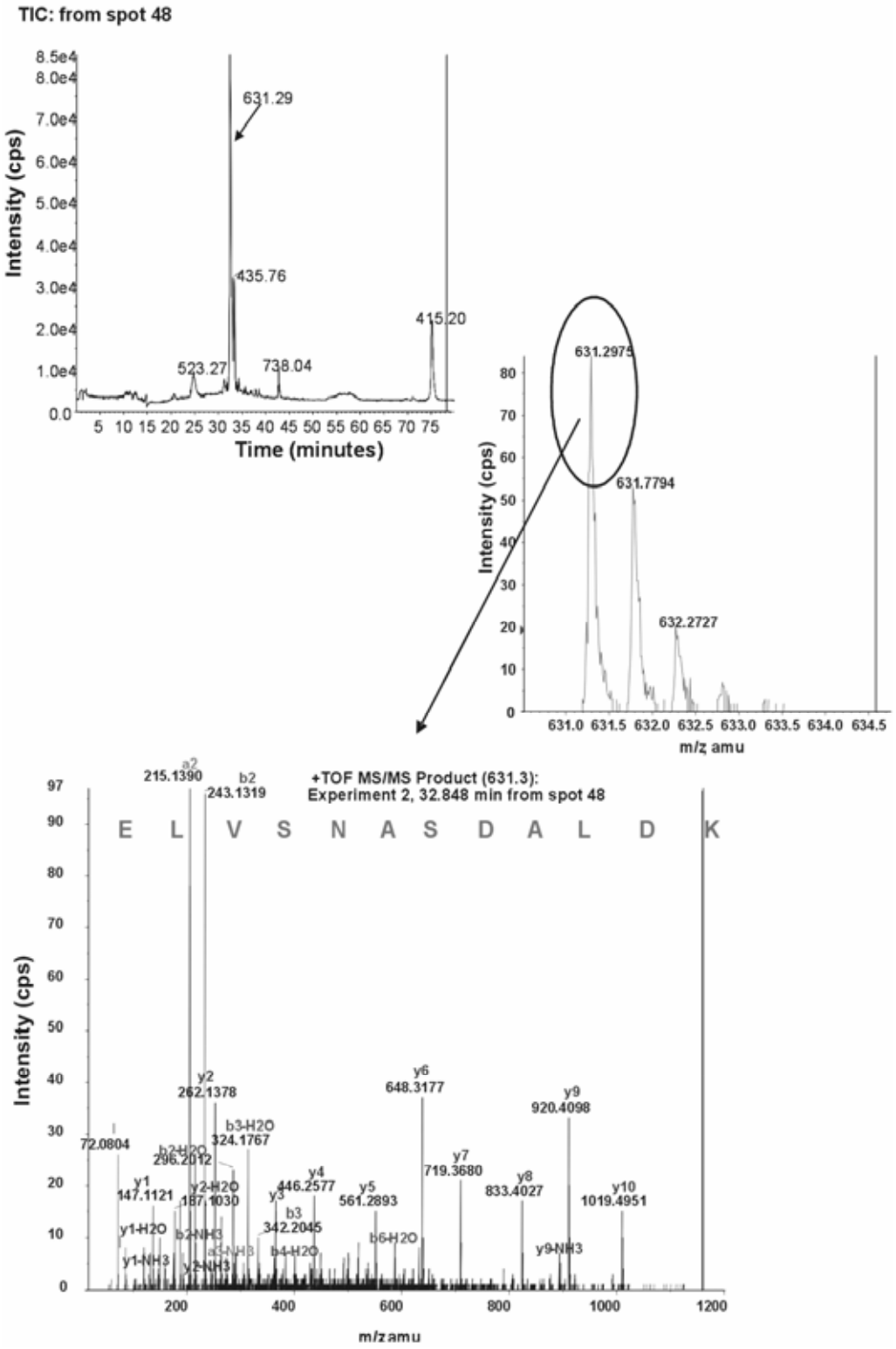

Fig. 5. Example of a chromatogram of spot 48 (upper left), mass spectrometry (MS) full-scan at 32.797 min. (close-up of peak 631.2975) (middle), and the MS/MS of peak 631.2975 with b and y ions labeled and deduced amino acid sequence (lower left).

that these RGA belong to the NBS-LRR superfamily and the TIR-NBS-LRR subfamily and were further divided into 14 cluster-based classes. Based on cDNA cloning and reverse transcriptase-PCR analyses, 10 of these 14 classes were determined to be expressed in resistant seedlings of *Pinus monticola*. The work by Liu and Ekramoddoullah (2003) has provided a glimpse of the large and diverse NBS-LRR gene family that may be functional in conifers. Our results indicate that diverse homologs of this family (NBS-LRR) and the TIR-NBS-LRR subfamily are also present and function in *Pinus strobus*. It

Fig. 6. Amino-acid alignments of matching peptide sequences from MS-BLAST database searches. Query sequences are underlined and dashes (–) indicate separate tryptic peptides. Gray shading indicates aligned sequences. Peptides in bold indicate matches made with Pro-ID in addition to MS-BLAST. Annotation of protein matches are above each alignment.

Spot 765: H+-ATPase I subunit from *Pinus koraiensis* (Q85WS7)
-**EVEMLGDELR**-**TLNQNLGLMR**
-EVEMIGDEIR-TINQNIGLFR

Spot 765: NADH dehydrogenase subunit from *Phymaturus punae* (Q71SE3)
-AMASSLLSGWGG-PTTLLVAPM-
-AMTSTLIGGWGG-PTTLLILPM-

Spot 765: Disease resistance protein (TIR-NBS-LRR): *A. thaliana A. thaliana* (Q94AG2)
-SGLQLLDLSNNR-**EVEML**-
-SNIQLLDLSNNR-EVEMI-

Spot 765: Photosystem II oxygen-evolving complex protein 2 from *Pinus monticola* (Q7M1Y8)
-**GEAANVMGAPK**-
-GEAANVFGAPK-

Spot 770: ATP-dependent protease proteolytic subunit from *Pinus contorta* (Q85X43)
-**VMIHQPASSYY**-**AAEMHNEAK**-**APTEADATWVDLYNR**-**DLDRDVFMSA**-**AYGIVDTV**-**TDNPEEVL**-
-VMIHQPASSYY-AADFHNESK-APGDEDATWVDLYNR-DLNRDVFMSA-AYGIVDVV-TGQPEEVI-

-WYVQAELVNGR-
-WYVQAELVNGR-

Spot 770: 2-Cys peroxiredoxin from *Pisum sativum* (Q93X25)
-**GLFIIDKEGVIQHSTINN**-**EGVIQHSTINNLAIGR**-FGVLLADQGLALR-SIPNGPSAL-
-GLFIIDKEGVIQHSTINN-EGVIQHSTINNLGIGR-YGVLIPDQGIALR-SIPNAPNSL-

Spot 770: LRR protein from *Trypanosoma brucei* (Q7YW17)
-**CSDGLKGLNALGTLPR**-KGLNALGTLP-WVDLYN-
-CSGGLKGLNALGTLPR-KGIETLGTLP-WVDLNN-

Spot 785: Cytosolic phosphoglycerate kinase from *Nicotiana tabacum* (Q42961)
-**FAVGTESIAK**-**TFSEALDTTK**-ISDVLSK-
-FAVGTEAIAK-TFNDALDTTK-IKDLLSK-

Spot 785: Putative LRR disease resistance protein/transmembrane receptor kinase from *A. thaliana* (NP_178230)
-**VVSLSIPR**-
-VVSLSIPR

Spot 959: Cytochrome c oxidase subunit II *Polytomella* sp. 'Pringsheim 198.80'(Q9AQY5)
-**SPTVIALR**-**VVEALSPR**-
-SPTVIALR-VVEAISPR-

Spot 963: Acid phosphatase from *Archaeoglobus fulgidus* (O29320)
-**SLSIMEPIR**-
-SLSIMEPIR-

Spot 963: Putative LRR disease resistance protein/ transmembrane receptor kinase from *A. thaliana* (NP_178230)
-**VVSLSIPR**-
-VVSLSIPR-

Fig. 6. *Continued from preceding page.*

would be useful to determine what differences exist between RGA in susceptible and resistant *Pinus strobus* (i.e., whether certain genes are absent from susceptible genotypes or if susceptibility is the result of repression or nonexpression of RGA).

The lack of a sequenced genome for the genus *Pinus* has limited the use of one or both high-throughput genomic or proteomic strategies for identifying genes and proteins that play a role in host defenses in pines. The strategy employed in this study was useful for identification of up-regulated proteins; however, this study should be expanded to include other candidate proteins not usually seen on 2-D gels, such as membrane proteins of low abundance proteins. Release of complete genomic sequences of loblolly pine (*Pinus taeda*) and other species will significantly improve throughput and accuracy of proteomic comparisons of spots from 2-DE gels by use of peptide mass fingerprinting (Dreger 2003), will define candidate genes that could be directly studied, and will allow the definition of regulatory sequences that may be common in genes defined as upregulated during *C. ribicola* infection.

Choosing an appropriate timepoint for sampling is difficult with this system, since very little is known about the early infection process with this host. In addition, symptoms do not appear until at least 3 wpi, even under optimum conditions. Frequently artificial inoculations are unsuccessful or infection frequency is low. Thus, sampling too early in the process would be arbitrary, since infected tissue cannot be detected and could be useless if infected samples are not chosen. The disease cycle takes several years and resistance may be expressed long after the initial infection takes place. This resistance mechanism has been described as a "slow rusting response" in Western species of pine (Kinloch et al. 1999). In this study, 4 weeks was chosen for two reasons. First, symptoms were clearly visible at 4 weeks, with differences in spot sizes between susceptible and resistant seedlings evident. This would ensure that proteins from infected tissue would be present. Second, the histological sections from 4 weeks also showed evidence of an HR-like response, similar to those observed previously by Jurgens and associates (2003).

This is the first example of putative disease-resistance proteins being identified in the unsequenced genome of *Pinus strobus*. These results provide a snapshot of some of the differences, at the molecular level, between WPBR-susceptible and -resistant genotypes of *Pinus strobus*. Although further studies are needed to determine if the proteins identified are responsible for the phenotypic responses observed in inoculated seedlings, the data reported here provide a foundation for better understanding of the molecular basis for disease resistance in *Pinus strobus*. In addition, it would be useful to obtain the gene sequences that encode these proteins in order to complete phylogenetic comparisons. Future direction will also examine whether regulatory elements of these putative rust-resistant genes may have a common regulatory mechanism that can be exploited for resistant-gene expression.

MATERIALS AND METHODS

Seedling inoculations.

Pinus strobus seedlings (60 per seed source) from controlled self-crosses of resistant (P327) and susceptible parents (H111) growing at the U. S. Forest Service Oconto River Seed Orchard, (White Lake, WI, U.S.A.) were grown for five months in greenhouse conditions under artificial lights. The seedlings were transferred to a growth chamber and were inoculated with *C. ribicola* using the protocol described by Jurgens and associates (2003). After 7 days, the seedlings were transferred back to the greenhouse and were grown under the same conditions as prior to inoculation. Mock-inoculated seedlings were treated the same as inoculated, except moistened cloth was substituted for telia-bearing *Ribes* leaves. When spots began to appear at 4 wpi, histological sections (Fig. 1) of infected (symptomatic) needles from susceptible and resistant seedlings were obtained and were stained with Schiff's reagent (Jurgens et al. 2003) to document hyphal penetration of host tissues.

Total protein extractions.

At 4 wpi, 0.2 g of primary needles from 12 seedlings each of inoculated and mock-inoculated seedlings of H111 and P327 were transferred immediately to sterile mortars with liquid nitrogen. Needles were selected from infected seedlings that had developed spots typical of infection by *C. ribicola*. The tissue was ground to a fine powder using a pestle and was transferred to sterile 2.0-ml microcentrifuge tubes on ice. Total protein extractions were performed, using the ReadyPrep total protein extraction kit (BioRad, Inc, Hercules, CA, U.S.A.), following manufacturer's instructions. Carrier ampholytes (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) were added at a concentration of 0.2% wt/vol. In addition, protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, U.S.A.) was added to the samples, according to manufacturer's instructions, to reduce the risk of protease degradation. The protein concentrations were assayed using the RC/DC protein assay (BioRad, Inc.) according to manufacturer's instructions. Five 10-fold serial dilutions of bovine serum albumin (0.2 mg/ml) were used to create a standard curve. Sample dilutions were loaded into 96-well microtiter plates, and spectrometric measurements were recorded at 750 nm with a Biotek Industries microplate spectrophotometer. Samples were further subjected to the ReadyPrep 2-D cleanup kit (BioRad, Inc.) according to manufacturer's instructions. Samples were stored at –80°C until they were used for 2-DE analysis.

2-DE.

2-DE was performed on duplicate gels for each sample according to the method of O'Farrell (1975). Isoelectric focusing was carried out in glass tubes with an inner diameter of 3.5 mm, using 2% ampholines (pH 4 to 8) (Gallard-Schlesinger Industries, Inc., Garden City, NY, U.S.A.) for 20,000 volthours. The pH gradient plot for these ampholines was determined using a surface-pH electrode. An IEF internal standard, tropomyosin (50 ng), was added to each sample prior to loading. Tropomyosin shows two polypeptide spots of similar pI; the lower spot has a molecular weight of 33,000 and pI of 5.2 (Fig. 2)

Tube gels were subjected to equilibrium in sodium dodecyl sulfate (SDS) sample buffer (10% wt/vol glycerol, 50 mM dithiothreitol, 2.3% wt/vol SDS, and 0.0625 M Tris, pH 6.8). Tube gels were then sealed to the top of a stacking gel that overlays a 10% acrylamide slab gel (0.75 mm thick) with 0.5% agarose in running buffer. SDS slab gel electrophoresis was carried out for approximately 5 h at 25 milliampere per gel. The following proteins (Sigma Chemical Co., St. Louis) were added as molecular weight standards to the agarose sealing the tube gel to the slab gel: myosin (222,000 Da), phosphorylase A (94,000 Da), catalase (60,000 Da), actin (43,000 Da), carbonic anhydrase (29,000 Da), and lysosyme (14,000 Da). The standards appear as horizontal lines on the Coomassie blue-stained 10% acrylamide slab gels. The gels were dried between transparent sheets and stored at room temperature.

Molecular weight and pI measurements.

The pI measurements are approximate, being based on the pH gradient plot for these ampholines for conditions of 9 M urea and room temperature of 22°C. The molecular weight and

pI values for each spot are determined from algorithms applied to the reference image using Progenesis Discovery software (version 2003.03, Nonlinear Technology, Grand Rapids, MI, U.S.A.).

Computational gel comparisons.

Duplicate gels from each sample were scanned with a laser densitometer (Model PDSI; Molecular Dynamics Inc., Sunnyvale, CA, U.S.A.). The scanner was checked for linearity prior to scanning with a calibrated Neutral density filter set (Melles Griot, Irvine, CA, U.S.A.). Images were analyzed using Progenesis Discovery software such that all spots were outlined, matched, and quantified by volume. Manual checking of the spot finding and matching functions was done, and when necessary, spots were determined and rematched manually. Average gel images were analyzed with the Progenesis software.

Normalized spot percentages from average gels were compared. Normalized spot percentage is equal to spot integrated density (volume) expressed as a percentage of total density of all spots measured on a gel to compensate for any loading or staining differences between gels. Differential expression is defined by the fold-percentage that the average gel image spots differ from that of the spot's matched counterpart in a comparison average gel. For example, if the spots both have the same spot percentage, the difference field will display as 1.000; if the spot in the comparison gel has a percentage twice as large, the difference field will display 2.000, indicating twofold upregulation; likewise, if the spot in the comparison gel has a value half as large, the difference field will display –2.000, indicating twofold downregulation.

Excision of gel spots and in-gel digestion.

Proteins were aseptically excised from the gels manually and were subjected to tryptic digestion (Schevchenko and Schevchenko and Schevchenko 2001), on the Genomic Solution ProPrep robot (Ann Arbor, MI, U.S.A.) in the Department of Biochemistry, Molecular Biology and Biophysics' Proteomic Analysis Core facility, University of Minnesota. Briefly, gel slices in 96-well trays were subjected to two series of dehydration and hydration steps by the addition, incubation, and removal of acetonitrile (ACN) followed by the addition, incubation, and removal of 25 mM NH_4HCO_3. Gel slices were then subjected to reduction in the presence of 10 mM dithiothreitol (DTT) and 25 mM NH_4HCO_3 at 60°C for 30 min. The DTT solution was aspirated, and a 55 mM iodacetamide and 25 mM NH_4HCO_3 solution was added for 30 min at 25°C. The iodacetamide solution was aspirated, followed by two series of dehydration and hydration steps as above. Gel slices were then subjected to tryptic digestion with 12 ng of trypsin per microliter (Promega, Madison, WI, U.S.A.) in 25 mM NH_4HCO_3, 5 mM $CaCl_2$ at 37°C for 10 h. The reaction was stopped with the addition of formic acid to a final concentration of 0.1% (wt/vol). The solution was then transferred to a siliconized 1.5-microcentrifuge tube. The gel slice was subjected to extraction with the addition of ACN for 10 min, followed by addition of 0.1% formic acid for 10 min. Solutions from a single plug were pooled, frozen, and concentrated in a speed vacuum.

Electrospray mass spectral analysis.

Samples were rehydrated in loading buffer in preparation for liquid chromatography (LC) MS/MS (30 µl of 98:2 water/ACN, 0.1% triflouroacetic acid). An LC Packings (LCP) Famos auto sampler (Dionex Company, Sunnyvale, CA, U.S.A.) aspirated 27.5 µl of the peptide dilution into a 100-µl sample loop, using the Famos µl-pickup injection mode and load buffer as the transfer reagent. An LCP Switchos pump was used to concentrate and desalt the sample on an LCP C18 nano-precolumn (0.3 mm internal diameter × 5 mm length) with load buffer. The precolumn was switched in-line with a capillary column, and peptides were eluted at 350 nl per minute, using an LCP Ultimate LC system. The capillary column (100 µm internal diameter) was packed in-house to 12-cm length with 5 µm, 20-nm pore size C^{18} particles (Michrom BioResources, Auburn, CA, U.S.A.), as described by Mosely and associates (1997). Peptides were eluted with a linear gradient over 44 min, starting with 100% solvent A (95:5 water/ACN, 0.1% formic acid), to a final solvent B (5:95 water/ACN, 0.1% formic acid). The LC system was obtained online from Applied Biosystems, Inc. (ABI, Inc., Foster City, CA, U.S.A.) QSTAR Pulsar quadrupole-time of flight (TOF) mass spectrometer (MS), which was equipped with Protana's (Odense, Denmark) nano-electrospray source. An electrospray voltage of 2.250 kV was applied distal to the analytical column. The TOF region acceleration voltage was 4 kV, and the injection pulse repetition rate was 6.0 kHz. The $[M + 3H]^{3+}$ monoisotopic peak at 586.9830 *m/z* and $[M + 2H]^{2+}$ monoisotopic peak at 879.9705 *m/z* from human renin substrate tetradecapeptide (Sigma-Aldrich, Inc.) were used for external calibration. As peptides eluted from the column, they were focused into the mass spectrometer and product ion spectra were collected in an information-dependent acquisition (IDA) mode. IDA mode settings included continuous cycles of one full scan TOF MS from 400 to 1,100 *m/z* (1.5 s) plus three product ion scans from 50 to 2,000 *m/z* (3 s each). Precursor *m/z* values were selected from a peak list automatically generated by Analyst QS software (ABI, Inc.) from the TOF MS scan during acquisition, starting with the most intense ion.

Sequence analysis.

Mass spectra were analyzed using the QSTAR Analysis Software package. De novo sequences (Shevchenko and Chernushevich 1997) were checked for accuracy by examining ion fragmentation. Product ion mass spectra were searched using ProID (ABI, Inc.) against the National Center for Biotechnology Information's nonredundant database (April 22, 2004) for protein identification. The following search parameters were used: 1 trypsin missed cleave site; peptide and product ion tolerance = 0.35 Da; custom amino acids, J = carbamidomethyl cysteine, O = singly oxidized methionine; additional modifications included in the search were pyroglutamic acid, *O*-phosphorylation of S,T,Y, deamidation of Q and N. MS-BLAST database searches were conducted using de novo sequences according to the protocol described by Shevchenko and associates (2001). Final sequences were deposited into the UniProtKB/Swiss-Prot Protein database.

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