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Proteomic and genetic approaches to identifying defence-related proteins in rice challenged with the fungal pathogen *Rhizoctonia solani*

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SUMMARY

Sheath blight, caused by the fungus *Rhizoctonia solani*, is a major disease of rice world-wide, but little is known about the host response to infection. The objective of this study was to identify proteins and DNA markers in resistant and susceptible rice associated with response to infection by *R. solani*. Replicated two-dimensional polyacrylamide gel electrophoresis experiments were conducted to detect proteins differentially expressed under inoculated and non-inoculated conditions. Tandem mass spectra analysis using electrospray ionization quadrupole-time of flight mass spectrometry (ESI Q-TOF MS) was carried out for protein identification with the NCBI non-redundant protein database. Seven proteins were increased after inoculation in both susceptible and resistant plants. Six of the seven proteins were identified with presumed antifungal, photosynthetic and proteolytic activities. An additional 14 proteins were detected in the response of the resistant line. Eleven of the 14 proteins were identified with presumed functions relating to antifungal activity, signal transduction, energy metabolism, photosynthesis, molecular chaperone, proteolysis and antioxidation. The induction of 3-β-hydroxysteroid dehydrogenase/isomerase was detected for the first time in resistant rice plants after pathogen challenge, suggesting a defensive role of this enzyme in rice against attack by *R. solani*. The chromosomal locations of four induced proteins were found to be in close physical proximity to genetic markers for sheath blight resistance in two genetic mapping populations. The proteomic and genetic results from this study indicate a complex response of rice to challenge by *R. solani* that involves simultaneous induction of proteins from multiple defence pathways.

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INTRODUCTION

Rice (*Oryza sativa* L.) is considered a model crop plant owing to its importance world-wide as a food source (Sasaki and Burr, 2000), a small diploid genome suitable for genetic and molecular analyses (Devos and Gale, 2000), and available transgene technology (Hiei *et al*., 1994). Completion of the draft rice genome sequence (Goff *et al*., 2002) has encouraged researchers to focus on identity, function and regulation of genes on a genome-wide scale. Because proteins are key components of structure and play vital roles in the cell, proteomic analysis can ideally provide direct functional information by exploring the global expression patterns of proteins in various states (Rakwal and Agrawal, 2003). Rice proteomics research has made considerable progress recently in providing functional information of proteins expressed in the various developmental stages, tissues, cells, and abiotic and biotic stress environments (Komatsu and Tanaka, 2004). For example, a recent proteomic study using two-dimensional polyacrylamide gel electrophoresis (2-DE PAGE) and multidimensional protein identification technology (MudPIT) detected over 25 000 peptides and 2500 unique proteins belonging to various biochemical pathways from rice leaf, root and seed sources (Koller *et al*., 2002). A rice anther proteome map has been constructed that contains over 4000 protein spots presented in 2-D gels within the pI range 4–11 with molecular weights of 6–122 kDa, corresponding to ~10% of the rice genome (Imin *et al*., 2001). A comprehensive rice proteome database was recently constructed that contains 11 941 identified proteins and 21 reference proteome maps based on 2-DE PAGE, including various rice tissues and subcellular compartments (Komatsu and Tanaka, 2004). The primary focus of rice proteomic research to date has centred on detection and characterization of proteins in response to biotic and abiotic stresses. Rakwal and Komatsu (2000) investigated rice seedlings after treatment by jasmonic acid (JA), and detected induction of various proteins including pathogenesis-related proteins (PR-proteins) involved in stress response, supporting a role of JA in the rice

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defence system. Shen *et al*. (2003) monitored changes in the rice leaf sheath proteome responding to physical wounding and observed decreased proteins associated with protein folding, and oxygen transfer while also detecting induced proteins associated with protease inhibition, signal transduction, photosynthesis and abiotic stress. Konishi *et al*. (2001) observed that PR-5 was induced by infection of the leaf blade by the pathogen *Magnaporthe grisea*, and the level of ribulose-1,5-bisphospate carboxylase/ oxigenase (RuBisCO) proteins was modified depending on the specific amount of nitrogen fertilizer applied to the soil. S. T. Kim *et al*. (2003) reported induction of various proteins including rice PR proteins and salt-inducible proteins related to general stress responses and regulatory enzymes in suspension-cultured rice cells after inoculation with *M. grisea* and defence-inducing elicitors JA, salicylic acid (SA) and hydrogen peroxide (H_2O_2). Brassinosteroid (BR), the most well-known plant steroid hormone, has been reported to play a role in cold stress response of rice seedlings (Hotta *et al*., 1998), and in resistance to the pathogens *M. grisea* and *Xanthomonas oryzae* pv. oryzae (Nakashita *et al*., 2003).

Sheath blight disease, caused by *Rhizoctonia solani,* is a major constraint to high grain yield and quality in many rice-growing regions of the world (Marshall and Rush, 1980). *R. solani* is believed to secrete various toxins, the most notable of which is the RS phytotoxin, a carbohydrate molecule containing glucose, mannose, *N*-acetylgalactosamine and *N*-acetylglucosamine (Vidhyasekaran *et al*., 1997). General symptoms of sheath blight include necrotic, dark, reddish-brown, elliptical or oval-shaped areas on the leaf sheath, leaf blade and culm (Lee and Rush, 1983). Modern semi-dwarf varieties in the US and elsewhere are normally grown at high densities that create a favourable environment for development of sheath blight (Lee and Rush, 1983). Most varieties grown around the world are susceptible to *R. solani*, although moderate to high levels of tolerance have been reported (Pan *et al*., 1999). The somaclonal mutant LSBR-5 (Xie *et al*., 1992), selected from the sheath blight-susceptible variety Labelle (Marshall and Rush, 1980), has shown high levels of resistance both in greenhouse and field studies (Xie *et al*., 1992). Genetic control of resistance in LSBR-5 was reported to be controlled by a single recessive gene (Xie *et al*., 1992). Previous genetic mapping studies have identified quantitative trait loci (QTL) and individual markers associated with sheath blight resistance in a population of recombinant inbred lines (RILs) (Pinson *et al*., 2005; Pinson, Oard and Capdevielle, unpublished data).

Even though sheath blight disease exerts a substantial impact on rice production in many regions of the world, limited information is available on response to infection by *R. solani* at the protein or genetic level. The primary objectives of this research were to monitor the global defence response of rice leaf sheath proteins to *R. solani* infection and to identify candidate proteins and associated DNA markers that can serve in future studies of rice–fungal interactions and in development of new, disease-resistant varieties. We also report here for the first time that 3-β-hydroxysteroid dehydrogenase was induced in resistant rice after infection by *R. solani*, suggesting that this enzyme may play a role in synthesis and regulation of steroids associated with disease resistance.

RESULTS

2-DE PAGE analysis and protein identification

Approximately 1000 protein spots stained with Sypro-Ruby (Bio-Rad, Hercules, CA) were reproducibly resolved on all 24 2-D gels used in the comparison analysis, which consisted of three subreplications of a protein sample, two treatments of infection (inoculation vs. non-inoculation), two biological replications and two rice strains (resistant vs. susceptible). Protein spots were reproducibly resolved across all gels, resulting in similar protein spot locations across replications (Fig. 1). With PDQUEST (Bio-Rad) image analysis software, 21 protein spots were detected whose relative abundance varied in the response of the resistant LSBR-5 (Fig. 2) or susceptible Labelle to *R. solani* infection. Seven increased protein spots were detected in the response of susceptible Labelle, and 14 additional protein spots (11 increased, three decreased) were detected in the response of resistant LSBR-5 (Fig. 3). Seventeen out of 21 detected protein spots were identified through the MASCOT MS/MS ion search engine with MS/MS spectra produced by Q-TOF (Waters, Milford, MA). At least two peptides were identified from MS/MS spectra for protein identification, except protein spots 8, 9, and 14, for which a single peptide was identified for each one consisting of at least ten amino acid residues (Tables 1 and 2).

Six of the seven identified proteins whose relative abundance varied significantly in the resistant and susceptible lines are detailed in Table 1 with protein name, NCBI accession number and identity scores. For example, β-1,3-glucanase was increased in both the resistant and the susceptible lines, but the protein abundance ratio for the inoculated/non-inoculated treatments in the mutant line was twofold greater than the corresponding treatments applied to the susceptible plant line. In addition, the relative amount of protein detected in the inoculated resistant line was 2.5–4-fold greater when compared with the inoculated Labelle or non-inoculated LSBR-5. No differences in protein amounts were observed between non-inoculated LSBR-5 and non-inoculated Labelle. A similar treatment response and relative protein abundance pattern were observed for the 20S proteasome β-subunit and the 26S proteosome non-ATPase regulatory subunit. Relative abundance of three distinct RuBisCO large subunits and a precursor were detected in the inoculated LSBR-5 at 2–3-fold higher levels than in the corresponding susceptible Labelle treatments.

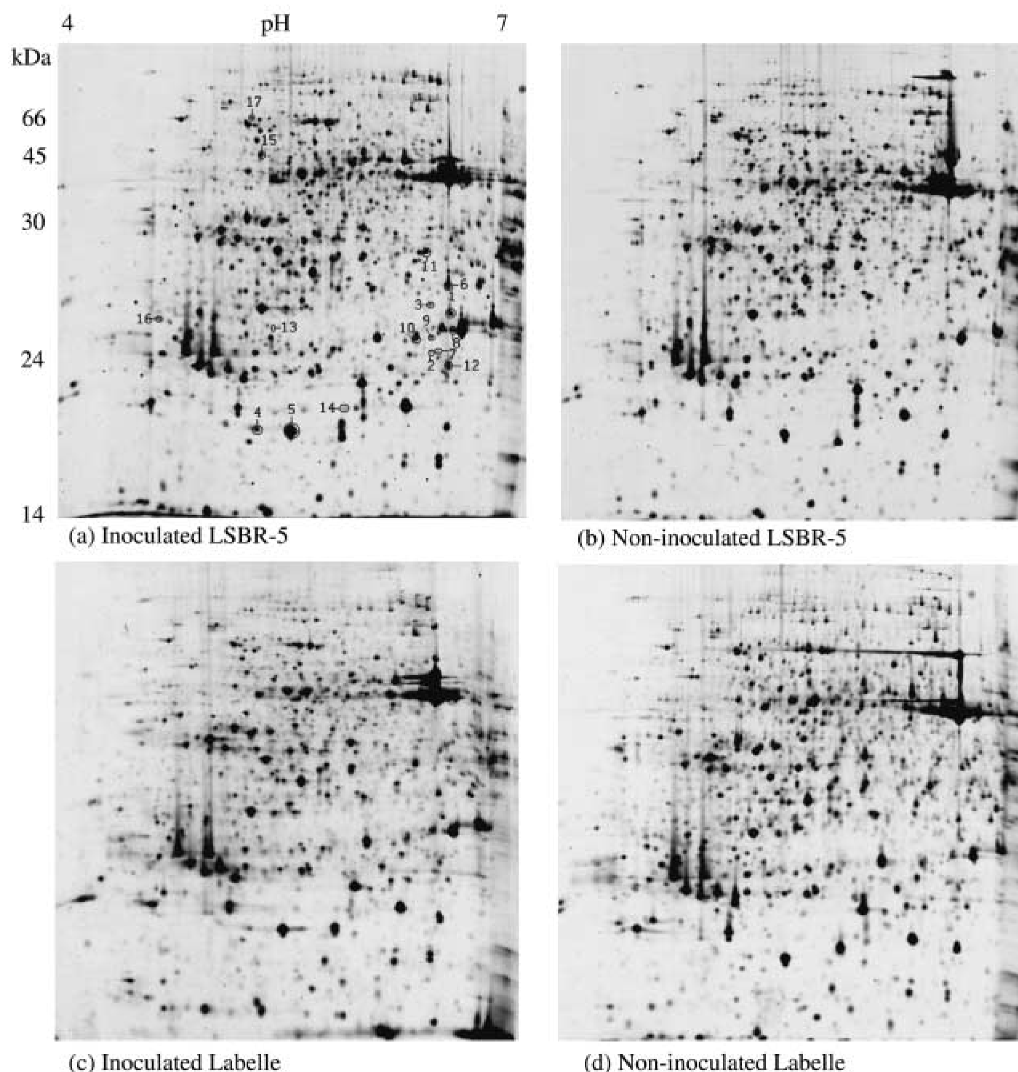

Fig. 1 Sypro-Ruby-stained 2-DE PAGE gels of rice leaf sheath proteins extracted from inoculated and non-inoculated resistant and susceptible lines. Proteins were separated with an IPG strip, pH 4–7, and 12% linear polyacrylamide LDS-PAGE. Gel images were acquired with an FX scanner (Bio-Rad). Circled proteins were identified by ESI-Q-TOF mass spectrometry. Spot numbers were assigned in an arbitrary fashion.

The relative abundance of 11 of 14 identified proteins that varied only in response of the resistant line to infection by *R. solani* is given in Table 2. The plant steroid-related 3-β-hydroxysteriod dehydrogenase/isomerasae (3-β HSD) was detected at threefold higher levels in inoculated vs. non-inoculated LSBR-5 and twofold higher levels in inoculated resistant vs. susceptible lines. No significant differences were found between infected vs. non-infected Labelle or non-inoculated treatments of the two lines. Three- to fivefold higher levels of two chitinase isoforms were detected in the inoculated vs. non-inoculated treatments of LSBR-5 and at twofold higher levels in the infected resistant vs. infected susceptible lines. RuBisCO large subunit and RuBisCO activase were induced at 4–5-fold higher levels in the inoculated vs. non-inoculated LSBR-5 and at threefold higher levels in the inoculated resistant vs. susceptible line. Similar treatment responses were detected for glyceradehyde 3-phosphate dehydrogenase (GAPDH) α subunit, 20S proteasome α-1 subunit and stromal ascorbate peroxidase (SAPX). Decreased protein spots were detected with infected LSBR-5 for putative chaperonin 60 beta precursor, 14-3-3-like protein and endosperm lumenal binding protein. Relative amounts of these proteins were at less than twofold lower levels in inoculated resistant LSBR-5.

Genetic and physical map of markers and proteins for response to *R. solani*

QTL regions and molecular markers previously mapped to three chromosomes associated with sheath blight resistance among a

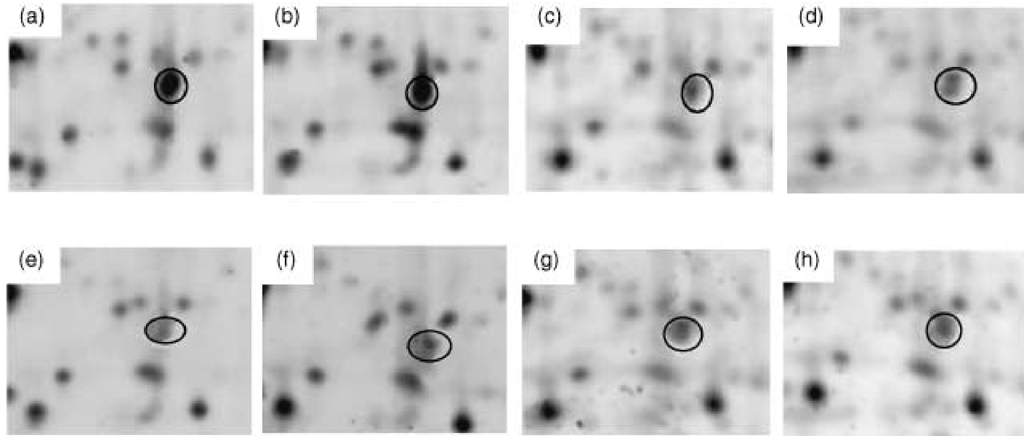

Fig. 2 Relative abundance of alpha 1 subunit of 20S proteasome detected in inoculated and non-inoculated LSBR-5 and Labelle. (a,b) Gels of inoculated LSBR-5 from two independent biological replications. (c,d) Gels of inoculated Labelle from two independent biological replications. (e,f) Gels of non-inoculated LSBR-5 from two independent biological replications. (g,h) Gels of non-inoculated Labelle from two independent biological replications.

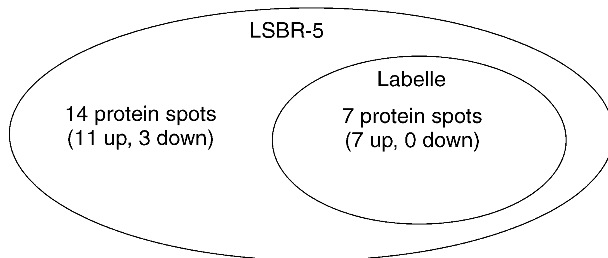

Fig. 3 Venn diagram of increased or decreased proteins in response of resistant LSBR-5 and susceptible Labelle infected by *R. solani*.

population of RILs (Pinson *et al.*, 2005) and F_4 families (Li *et al.*, 1995) are shown in Fig. 4. Marker alleles detected by discriminant analysis and step-wise regression (Pinson, Oard and Capdevielle, unpublished data) were included relative to the genetic regions of QTL and to the physical location of four proteins detected in this study. The QTL and discriminant/step-wise DNA markers mapped to the same or nearby regions for sheath blight resistance from two populations on chromosomes 2, 3 and 5. Based on release 3 of the TIGR pseudomolecule genomic database (http://www.tigr.org), the gene encoding SAPX detected in this study was located at a position within a previously defined QTL region (Li *et al.*, 1995) on chromosome 2 (Fig. 4) and separated by ~429 000 bp or ~2 cM from the marker C624x, selected by discriminant analysis and step-wise regression (Pinson, Oard and Capdevielle, unpublished data). Genes encoding the chitinase and GAPDH enzymes detected in this study were found at positions on chromosome 3 within QTL regions identified in two separate populations (Li *et al.*, 1995; Pinson, Oard and Capdevielle, unpublished data). The two genes were located ~200 000–430 000 bp or 1–2 cM from the marker RG348, selected both by discriminant and by step-wise regression methods to be associated with sheath bight resistance (Pinson, Oard and Capdevielle, unpublished data). Finally, the phenotypic marker *gl-1* (glabrous leaf), selected by discriminant and step-wise regression methods, mapped 9 cM from the marker RG556 on chromosome 5 that in turn was located ~105 000 bp or < 1 cM from 3-β HSD identified in this study.

DISCUSSION

Comparison between response of resistant LSBR-5 and response of susceptible Labelle to *R. solani*

Unlike other proteomic research on plant response to pathogens, this study was focused on the proteome response of rice leaf sheath adjacent to the infected areas. Although the direct interaction and response against the infection occurs in the infected area, it can be impossible to distinguish the response between rice proteome and *R. solani* proteome when the proteins from *R. solani* have homology with rice proteins owing to the fact that the protein and nucleotide database of *R. solani* is limited and the protein identification method with MS technology used in this study is absolutely dependent on the protein and nucleotide database. However, with the 2-DE proteomic approach on the rice leaf sheath adjacent to the infected area, we were able to detect reproducible proteome differences between the host response of the resistant mutant line LSBR-5 and that of the susceptible Labelle. The study revealed that seven out of the 21 identified proteins showed significant variation in relative abundance after pathogen challenge of the resistant LSBR-5 and the susceptible Labelle. A common stress response system appeared to be activated in both resistant and susceptible lines, but the resistant LSBR-5 line produced significantly larger amounts of

Table 1 Identified proteins whose relative abundance varied significantly in the resistant line LSBR-5 and the susceptible line Labelle with infection by *R. solani*.

Spot no.	Name	Accession no.	No. of peptides*	Sequence coverage† (%)	Score‡	Ratio of relative abundance§			
						IL/NL¶	I5/N5**	I5/IL	N5/NL
1	β-1,3-glucanase [*Oryza sativa*]	gi|4884530	4	12	253	2.16 ± 0.07	3.90 ± 1.20	2.50 ± 0.27	NS††
2	20S proteasome beta subunit [*Oryza satita* (*japonica* cultivar-group)]	gi|50933089	2	7	83	1.53 ± 0.05	3.28 ± 1.13	1.50 ± 0.05	NS
3	Putative 26S proteasome non-ATPase regulatory subunit 14 [*Oryza sativa* (japonica cultivar-group)]	gi|54287494	4	16	142	2.28 ± 0.26	4.43 ± 1.26	2.39 ± 0.52	NS
4	Rubisco large subunit	gi|476752	3	9	190	1.68 ± 0.01	2.11 ± 0.05	2.27 ± 0.01	NS
5	Rubisco large subunit	gi|476752	3	9	113	2.00 ± 0.14	5.16 ± 1.13	2.07 ± 0.87	NS
6	Ribulose-bisphosphate carboxylase (EC 4.1.1.39) large chain precursor–rice chloroplast [*Oryza sativa*]	gi|7436576	3	9	128	2.03 ± 0.48	4.13 ± 0.98	2.30 ± 0.18	NS

*Number of peptides identified from MS/MS spectra.
†Percentage of sequence coverage of identified peptides for protein.
‡Score = −10 × log(*P*), where *P* is the probability that the observed match with a given MS/MS spectra is a random event. Thus, a high score indicates identity or extensive homology.
§Ratios of relative abundance are presented as means ± SD ($n = 2$). The ratios were calculated by dividing the relative abundance of a protein spot in one treatment by the relative abundance of a protein spot in the corresponding treatment. A value greater than 1 represents an increased protein, whereas a ratio less than 1 represents a decreased protein.
¶IL, inoculated Labelle; NL, non-inoculated Labelle.
**I5, inoculated LSBR-5; N5, non-inoculated LSBR-5.
††NS, non-significant.

those proteins described in Table 1. All six of these identified proteins have been previously reported as being involved in disease response (Basset *et al*., 2002; Bera and Purkayastha, 1997; Konish *et al*., 2001), which suggests a common baseline defence strategy for different host–parasite interactions. For example β-1–3 glucanase, which enhances antifungal activity by degrading the fungus cell wall, was increased to a higher level in the resistant vs. the susceptible line. The phenomenon of induction of defence-related proteins was previously reported in the interaction between *Aradopsis thaliana* and the fungus *Peronospora parasitica* (Maleck *et al*., 2000). In the current study, seven proteins detected in both responses were increased to a significantly higher level in LSBR-5 than in Labelle, and an additional 14 proteins (Table 2), whose quantities did not vary in the response for Labelle, showed significant quantitative variation in the response of LSBR-5. Proteins identified with higher induced levels in LSBR-5 than in Labelle were previously reported as being involved in disease reactions (Asada, 1992; Broglie *et al*., 1991; Finnie *et al*., 2002; Lorimer, 1996; Shen *et al*., 2003; Umeda and Uchimiya, 1994). From the different protein expression patterns detected in this study, it is hypothesized that the resistance of LSBR-5 resulted from the ability to produce greater amounts of defence proteins than the susceptible Labelle, and this ability was possibly controlled by key regulatory protein(s) acquired during *in vitro* somaclonal culture of the resistant LSBR-5 mutant.

Identified proteins in response of rice leaf sheath to *R. solani*

Results indicated that 3-β HSD was increased only in the resistant line. To our knowledge, this is the first report of induction of 3-β HSD in response of rice to pathogen infection. Mutations of 3-β HSD in humans have been associated with the onset of childhood disease via disruption of the cholesterol biosynthetic pathway (Konig *et al*., 2000) and with congenital adrenal hyperplasia (Rheaume *et al*., 1994). This enzyme was reported in plants to play a role in the formation of cardinolides in Grecian foxglove (*Digitalis lanata*) (Finsterbusch *et al*., 1999). Milkweed produces cardenolide, which is toxic to insects, in the leaf as a putative defence mechanism against sucking insect herbivores (Martel and Malcolm, 2004). The occurrence of 3-β HSD enzyme activity in *Solanum tuberosum* and *Nicotiana tabacum* plants, which are unable to form cardenolides, implies that this enzyme may be involved in other steroid metabolic functions (Seidel *et al*., 1990). In recent proteomic research of ginseng (*Panax ginseng*), 3-β HSD was reported to be induced by high light conditions (Nam *et al*., 2003). Results from our study suggest that 3-β HSD may be involved in the synthesis or regulation of plant steroids associated with host defence, but further investigations will be required to elucidate the exact role of this protein in the rice–*R. solani* interaction.

Table 2 Identified proteins whose relative abundance varied significantly only in response of resistant LSBR-5 with infection by *R. solani*.

Spot no.	Name	Accession no.	No. of peptides*	Sequence coverage† (%)	Score‡	Ratio of relative abundance§			
						IL/NL¶	I5/N5**	I5/IL	N5/NL
7	Putative 3-beta hydroxysteroid dehydrogenase/isomerase protein [*Arabidopsis thaliana*]. AAM61751	gi|34897418	3	14	213	NS	2.76 ± 0.03	1.95 ± 0.18	NS††
8	Stromal ascorbate peroxidase [*Oryza sativa* (*japonica* cultivar-group)]	gi|32879781	1‡‡	2	45	NS	5.41 ± 0.87	3.27 ± 1.27	NS
9	Putative chitinase [*Oryza sativa* (*japonica* cultivar-group)]	gi|55168113	1§§	4	55	NS	4.79 ± 2.44	2.77 ± 1.70	NS
10	Putative chitinase [*Oryza sativa* (*japonica* cultivar-group)]	gi|55168113	4	21	179	NS	2.91 ± 0.20	2.00 ± 0.30	NS
11	Glyceraldehyde 3-phosphate dehydrogenase A subunit [*Arabidopsis thaliana*]	gi|166702	5	10	168	NS	2.76 ± 0.60	1.87 ± 0.50	NS
12	Putative alpha 1 subunit of 20S proteasome [*Oryza sativa* (*japonica* cultivar-group)]	gi|50920359	4	15	173	NS	3.98 ± 0.98	2.44 ± 0.06	NS
13	Ribulose-1,5-bisphosphate carboxylase/ oxygenase activase [*Oryza sativa*]	gi|1778414	2	4	70	NS	3.91 ± 1.01	3.03 ± 0.94	NS
14	Ribulose bisphosphate carboxylase large chain (RuBisCO large subunit)	gi|6093918	1¶¶	2	53	NS	5.19 ± 0.10	3.12 ± 0.18	NS
15	Putative chaperonin 60 beta precursor [*Oryza sativa* (*japonica* cultivar-group)]	gi|34897924	10	21	687	NS	0.67 ± 0.10	1.94 ± 0.20	3.20 ± 0.20
16	14-3-3-like protein [*Oryza sativa*]	gi|7271253	4	17	190	NS	0.60 ± 0.06	NS	1.55 ± 0.08
17	Endosperm lumenal binding protein [*Oryza sativa*]	gi|2267006	9	16	299	NS	0.65 ± 0.01	1.82 ± 0.17	2.30 ± 0.30

*Number of peptides identified from MS/MS spectra.
†Percentage of sequence coverage of identified peptides for protein.
‡Score = $-10 \times \log(P)$, where P is the probability that the observed match with a given MS/MS spectra is a random event. Thus, a high score indicates identity or extensive homology.
§Ratios of relative abundance are presented as means ± SD ($n = 2$). The ratios were calculated by dividing the relative abundance of a protein spot in one treatment by the relative abundance of a protein spot in the corresponding treatment. A value greater than 1 represents an increased protein, whereas a ratio less than 1 represents a decreased protein.
¶IL, inoculated Labelle; NL, non-inoculated Labelle.
**I5, inoculated LSBR-5; N5, non-inoculated LSBR-5.
††NS, non-significant.
‡‡Amino acid sequences: KYAEDQEAFFKD.
§§Amino acid sequences: RVLVGVVASPEADRD.
¶¶Amino acid sequences: KLTYYTPEYETKD.

The 16 additional proteins identified in this study have been previously reported to be involved in antifungal activity, signal transduction, energy metabolism, photosynthesis, protein folding and degradation, signal transduction, and antioxidation (Asada, 1992; Broglie *et al.*, 1991; Finnie *et al.*, 2002; Lorimer, 1996; Shen *et al.*, 2003; Umeda and Uchimiya, 1994). For example, two different protein spots were identified as putative chitinases that were increased only in the resistant line. Chitinase, referred to as PR-3, catalyses the hydrolysis of β-1–4-linkage of the *N*-acetylglucosamine polymer of chitins, a major component of fungal cell walls (Lin *et al.*, 1995). β-1,3-glucanase was also found in the current study to be up-regulated to a higher level in the resistant vs. susceptible line after challenge by *R. solani*. β-1,3-glucanase, referred to as PR-2, is a hydrolytic enzyme commonly found in plants that hydrolyses β-1,3-linked glucans, the major component of fungal cell walls (Van Loon *et al.*, 1987; Yamaguchi *et al.*, 2002). Consistent with this study, Bera and Purkayastha (1997) showed that β-1,3-glucanases and chitinase were induced upon infection with *R. solani*. In addition to pathogen attack, β-1, 3-glucanase was reported to be induced by various defence-signalling molecules such as SA, methyl jasmonate and ethylene (Linthorst *et al.*, 1990; Mauch and Staehelin, 1989; Rickauer *et al.*, 1997). Chitinase and β-1,3-glucanase can synergistically inhibit fungal growth *in vitro*, and the coexpression of these two enzymes was shown to enhance resistance against *R. solani* significantly in transgenic rice (J. K. Kim *et al.*, 2003; Mauch *et al.*, 1988). This synergetic effect may have contributed to the high resistance levels of LSBR-5 evaluated in our proteomic research.

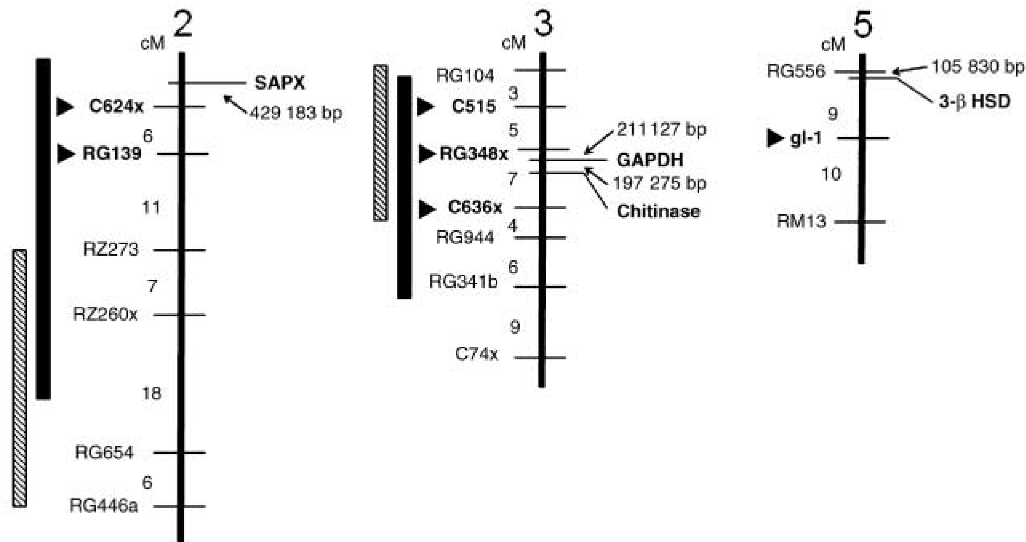

Fig. 4 Composite genetic and physical maps for QTL regions and DNA markers on chromosomes 2, 3 and 5 associated with sheath blight disease resistance and proteins detected in this study. Left side of chromosome shows QTL regions and genetic distance between markers in Kosambi map units (cM). Right side of chromosome shows proteins detected in this study and their physical distance to DNA markers in base pairs obtained from the Gramene website (http://www.gramene.org/). QTLs for sheath blight resistance identified by Li *et al.* (1995) using F_4-bulked lines from Lemont × Teqing cross. QTLs for sheath blight resistance identified by interval analysis (single and multi-QTL models) using 314 RILs from Lemont × Teqing cross (Pinson *et al.*, 2005). (■) QTLs for sheath blight resistance identified by Li *et al.* (1995) using F_4-bulked lines from Lemont × Teqing cross. (▨) QTLs for sheath blight resistance identified by interval analysis (single and multi-QTL models) using 314 RILs from Lemont × Teqing cross (Pinson *et al.*, 2005). ▶ Markers identified by Pinson, Oard and Capdevielle (unpublished data) and unpublished results using discriminant analysis and step-wise multiple regression. SAPX, stromal ascorbate peroxidase; GAPDH, glyceradehyde 3-phosphate dehydrogenase; 3-β HSD, 3-β-hydroxysteriod dehydrogenase/isomerasae; *gl-1*, phenotypic marker for glabrous leaf.

SAPX was increased only in the resistant line during our studies. Ascorbate peroxidase (APX) scavenges superoxide, hydroxyl radicals and singlet oxygen in the cytosol, chloroplast and mitochondria of higher plants. APX uses two molecules of ascorbate, the most important antioxidant substrate in plants, to reduce H_2O_2 to water (Asada, 1992). Decline of the SAPX protein level was detected in viral infected tobacco during programmed cell death (PCD), known as the hypersensitive response (HR) in plants (Mittler *et al.*, 1998). Suppression of APX proteins supposedly contributes to PCD by allowing H_2O_2 accumulation in the cells (Mittler *et al.*, 1998). The opposite results in our study, where SAPX proteins were increased in LSBR-5 and Labelle, suggest that the harvested leaf sheaths, without necrosis or injury of any kind, did not produce a PCD response.

A protein spot identified as homologous to GAPDH A subunit isoform from Arabidopsis was increased only in the resistant line. GAPDH plays an important role in glycolysis and gluconeogenesis by reversibly catalysing the oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, a high-energy intermediate used for the synthesis of ATP with a cosubstrate of NAD^+ (Umeda and Uchimiya, 1994). GAPDH is known to be induced by anaerobic conditions (Sachs *et al.*, 1996). GADPH was induced in potato leaves and stems by infection of the late-blight fungal pathogen *Phytophthora infestans*, by treatment of eicosapentaenoic acid (an elicitor found in *P. infestans*) or by exogenous SA, a signal molecule inducing systemic acquired resistance (SAR) (Laxalt *et al.*, 1996). These reports of GADPH induction under various environmental conditions, including abiotic and biotic stress, suggest that the GADPH metabolism pathway plays multiple roles in the plant cell or that related multiple defence pathways are regulated by similar signal transduction pathways (Laxalt *et al.*, 1996).

The 26S proteasome was increased in both the resistant and the susceptible lines with a higher expression ratio detected in the mutant vs. the susceptible wild-type. The 26S proteasome plays an essential role in the ubiqutin/26S proteasome-mediated ATP-dependent proteolysis system in which proteolysis is specific and strictly controlled (Vierstra, 2003). Plants respond to the various environmental changes by expressing new proteins as well as degrading regulatory proteins, damaged proteins, and proteins that may become useless in a new environment. The ability to switch from one physiological mode to another is essential for the growth of plants that cannot move away from unfavourable environments (Basset *et al.*, 2002). The induction of the 26S proteasome in both LSBR-5 and Labelle implies that the physiological status of both resistant and susceptible lines was shifted

to a defence mode. However, resistance of the somaclonal mutant is attributed to production of adequate amounts of the protein to mount a resistant response.

The 20S proteasome β subunit was increased in both the resistant and the susceptible lines of our study, where the expression ratio in the mutant line was higher than in the susceptible line. A putative α 1 subunit of 20S proteasome was increased only in the resistant line. The 20S proteasome, the proteolytic core of the 26 proteasome, is known to be involved in the degradation of proteins modified by oxidation (Grune *et al.*, 1995). In mammalian cells, the 20S proteasome has been shown to recognize and selectively degrade proteins damaged by oxidation, such as H_2O_2-modified haemoglobin (Giulivi *et al.*, 1994). The up-regulation of 20S proteasome in this study may be involved in the defence against oxidative stress, consistent with the induction of SAPX, an antioxidant protein, observed during our research.

The RuBisCO large subunits were increased in both the resistant and the susceptible lines of our study, where the expression ratio in the mutant line was higher than in the susceptible line. RuBisCO activase, the enzyme catalysing the activation of RuBisCO (Portis, 1990), was increased only in the resistant line. The level of RuBisCO proteins, a crucial enzyme for photosynthesis, is known to be reduced in infected plant cells because pathogens attack chloroplasts that lead to their degradation (Agrios, 1997). In recent rice proteomic research, however, RuBisCO proteins were reported to be increased in the rice leaf sheath by wounding stress (Shen *et al.*, 2003). The induced level of RuBisCO proteins in the response of LSBR-5 and Labelle may be explained by the unique area of harvested rice leaf sheath samples. Protein samples were extracted from the cells adjacent to the infected cells in our study to avoid contamination with fungal mycelia. Because the sampled leaf sheaths were not chlorotic and were green in colour, it is possible that the chloroplasts of the sampled cells were not yet directly affected by the infection, and photosynthetic activity was increased to compensate for loss in adjacent infected cells.

The 14-3-3 protein was decreased only in the resistant line after infection by the sheath blight fungus. The 14-3-3 protein is a phosphoserine-binding protein that regulates various target proteins by direct protein–protein interactions (Roberts, 2003). In plants, the 14-3-3 protein affects the activity of various enzymes and ion channels. For example, nitrate reductase and plasma membrane H+ pumping ATPase are inhibited and activated, respectively, by the direct interaction with 14-3-3 proteins (Comparot *et al.*, 2003; Kinoshita and Shimazaki, 1999). In the incompatible interaction in barley with an avirulent strain of the powdery mildew fungus, the 14-3-3 protein and plasma membrane H+ ATPase were induced in epidermal cell layers, site of the HR, which causes localized sudden death in infected host cells to prohibit further pathogen invasion (Finnie *et al.*, 2002). Zhou *et al.* (2000) proposed an HR model during the barley–powdery mildew interaction in which H+ ATPase is activated in the epidermal cell by the detection of the avirulent pathogen, causing acidification in the apoplast. This low pH is optimal for oxalate oxidase, an enzyme used in plants to generate H_2O_2. The down-regulation of the 14-3-3 protein in our study suggests that the response of LSBR-5 is not consistent with the barley HR model. However, the 14-3-3 protein may have actually been induced either before or after the time of leaf sheath sampling in our study.

The induction of SAPX and 20S proteasome in our study, presumably to reduce host cell oxidative stress, may have accompanied the down-regulation of the 14-3-3 protein. As pointed out by Wan *et al.* (2002), production and removal of H_2O_2 must be strictly controlled during SAR because H_2O_2 acts not only as an antimicrobial compound and a signal molecule to induce defence proteins, but also as a toxic molecule to host plants. Thus, down-regulation of the 14-3-3 protein at the 24-h sampling time was consistent with induction of SAPX and 20S proteasome in LSBR-5 to reduce toxic effects of H_2O_2.

The chaperonin 60 β precursor was decreased in this study only in the resistant line. Chaperonin is known to be a stress-related protein that binds particularly to denatured proteins to prevent degradation and to assist in protein refolding of ATP (Rochester *et al.*, 1986). In eubacteria and eukaryotic organelles, chaperonin 60 is presumably involved in numerous enzyme-folding functions (Lorimer, 1996). In plant chloroplasts, the level of chapronin 60, being involved in assembly of RuBisCO holoenzyme, is normally coordinated with that of RuBiSCO (Avni *et al.*, 1989). However Holland *et al.* (1998) reported that the accumulation of chapronin 60 was detected in *N. tabacum* seedlings by salt, cold or prolonged darkness whereas the RuBisCO large subunit was decreased. This negative correlation suggests the possible role of chapronin 60 in stress responses. In our study, a slight reduction of the putative chaperonin 60 beta precursor was detected with induction of RuBisCO proteins in the response of LSBR-5 to *R. solani*.

The luminal-binding protein (LBP) was found to be decreased only in the resistant somaclonal line. This protein is reported to be involved in folding and synthesis of defence-related proteins produced in the rough endoplasmic reticulum (ER) (Jelitto-Van Dooren *et al.*, 1999). In tobacco, LBP was induced rapidly in response to attack by *Erwinia carotovora*, reaching a maximum accumulation 2 h after inoculation. It is unknown how down-regulation of ER chaperones is related to the defence response in rice, but it has been reported that calreticulin in the endoplasmic reticulum, a Ca^{2+} binding protein that has molecular chaperone function, was down-regulated in the rice leaf sheath by wounding stress (Shen *et al.*, 2003).

Results from our proteomic study suggest that the response of rice to challenge by *R. solani* involved the recruitment of proteins from various defence pathways. Several of the proteins detected in the current study were previously reported to be expressed by

various pathogen treatments as well as various abiotic stresses, suggesting that some common response pathways exist in the rice defence system. Based on results from this study, the rice response to *R. solani* may be described where energy required to induce a defence was supplied by proteins involved in energy metabolism (GAPDH, RuBisCO), and the physiological status of infected rice was shifted to a defence mode via induction of the proteolytic enzymes 26S proteasome and 20S proteasome. The host response to infection was characterized by induction of the antioxidant protein SAPX accompanied by a slight reduction in the H_2O_2-inducing 14-3-3 protein that protected plant cells from oxidative stress. The antifungal proteins chitinase and glucanase were used directly to attack and degrade cell walls of *R. solani*.

Although we detected differences between the resistant and susceptible lines with the 2-DE PAGE approach, the proteome evaluated in our study was focused within a pH range of 4–7 and at a single time point 24 h post-inoculation. Multidimensional separation techniques such as MudPIT (Link *et al.*, 1999), coupled with multiple time points for tissue sampling, will be required in future studies to identify additional low-abundance, basic, hydrophobic or membrane-bound proteins associated with resistance to *R. solani.*

Comparative analysis between proteomics approach and genetic analysis

Results from our proteomic and genetic studies suggest that the identified proteins play a role(s) in the defence mechanism of rice when challenged by *R. solani*. Figure 4 shows the genomic location of genes encoding the induced proteins SAPX, chitinase, GAPDH and 3-β HSD, and their relationships among the identified QTL regions and selected DNA markers believed to be associated with sheath blight resistance. Consistency between the proteomic and genetic data obtained from this study enhances confidence in the identity of proteins involved in sheath bight resistance and demonstrates the potential value of combining different technologies to address questions not possible by a single approach. It will be of value to conduct further comparison analyses among all detected proteins in our study and various QTL for defence against biotic and abiotic stresses. Results from this study are the first to demonstrate a correspondence between genetic and proteomic approaches for resistance to *R. solani*, which supports the suggestion that proteomics can be combined with genetic analysis to provide 'a clue for explaining QTLs' (Prioul *et al.*, 1999).

EXPERIMENTAL PROCEDURES

Plant material and inoculation

Seeds from susceptible Labelle and resistant LSBR-5 were sterilized in 50% bleach, rinsed with water and geminated in Petri dishes at 28 °C in the dark. Three individual germinated seeds per line were placed in a single pot 16 cm in diameter and grown in the greenhouse under natural light conditions for 6 weeks before inoculation. Temperature of the greenhouse was ~30 °C during the day and ~22 °C at night. Six pots were prepared for each line, with three pots for the inoculated treatment and three for the non-inoculated treatment.

A modified inoculation method based on that of Singh *et al.* (2002) was used, in which *R. solani* strain LR 172 was grown on potato dextrose agar (DIFCO, Franklin Lakes, NJ) for 4 days at 28 °C in the dark to produce white, compact, immature sclerotia, ~1 mm in diameter. A rice leaf sheath from a 6-week-old plant at the late tillering stage was opened gently to expose a stem. A single sclerotium was placed beneath the leaf sheath and ~50 μL of sterilized water was added. Leaf sheaths of control non-inoculated plants were opened slightly, and sterilized water was added. After inoculation, plants were placed in a home-made humidifier chamber (130 cm long, 115 cm wide, 115 cm high, containing 150 L of water, covered with 0.03-mm-thick plastic) at 17:00 h for 24 h to maintain ~100% humidity on the leaves. To reduce plant stress caused by constant heat and humidity, the plastic was removed from the chamber the following morning at 08:00 h for 10 min.

Two separate experimental (biological) replications were carried out in this study where each replication consisted of four treatments: inoculated LSBR-5, non-inoculated LSBR-5, inoculated Labelle and non-inoculated Labelle. For each treatment, one pooled protein sample was created by extracting and combining proteins from treated leaf sheaths of 7–9 plants. The pooled protein sample was distributed equally among three aliquots, which served as subreplications of the pooled protein sample. Thus, this experimental design consisted of two biological replications, four treatments and three subreplications nested within the treatments.

Protein extraction and 2-DE PAGE

After 24 h of incubation in the plastic chamber, inoculated leaf sheaths showed typical disease lesions. To determine the appropriate tissue for harvest that did not contain mycelia of *R. solani*, inoculated leaf sheaths were stained in five separate experiments with Trypan Blue (Sigma, St Louis, MO) (Frye and Innes, 1998) that showed mycelia were not detected ~2 cm above the lesion formed 24 h after inoculation (data not shown). Therefore, to avoid contamination with *R. solani*, ~3 cm of leaf sheath tissue was harvested 2 cm above the lesion. The harvested leaf sheath samples were immediately placed on dry ice and then stored at –80 °C until protein extraction. The protein extraction method was based on that of Koller *et al.* (2002) with some modifications. Briefly, frozen leaf sheaths were ground in a mortar with liquid nitrogen and suspended in 10% TCA in acetone with 0.07% DTT

at –20 °C for 1 h, followed by centrifugation for 15 min at 35 000 ***g***. The pellets were washed once with ice-cold acetone containing 0.07% DTT at –20 °C for 1 h and centrifuged again for 15 min at 35 000 ***g***. This washing step was repeated 4–5 times until the supernatant was clear. The final precipitated pellets were freeze-dried in an AdVantage Freeze Dryer (VirTis, Gardiner, NY). A total of 10 mg of the dried powder was dissolved in 350 μL of buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% ampholytes (pH 3–10) and 0.7% DTT. The powder dissolved in the extraction buffer was gently shaken for 1 h, and then centrifuged for 30 min at 35 000 ***g*** at room temperature. The supernatant was distributed in 100-μL aliquots and kept at –80 °C until 2-D PAGE analysis. A total of 100 μg of protein, assayed by PlusOne™ 2-D Quant Kit (Amersham Biosciences, Piscataway, NJ), was loaded on to a 17-cm linear IIPG strip (Bio-Rad). The first dimensional electrophoresis (isoelectric focusing) was carried out with a Protein Isoelectric Focusing Unit (Bio-Rad) according to the manufacturer's instructions. The second dimensional electrophoresis was conducted on 12% polyacrylamide LDS (lithium-dodecyl sulphate) gels using Bio-Rad Protein II XL Gel Cell (Bio-Rad). The second electrophoresis running conditions were as follows: constant 16 mA for 30 min at 6 °C followed by constant 30 mA per gel until the BPB dye reached the bottom of the gel. The 2-D gels were stained with Sypro-Ruby (Bio-Rad), and images were acquired using an FX scanner (Bio-Rad).

Image analysis

Spot detection and matching analysis were conducted first with the PDQUEST program (Bio-Rad) and then manually checked a second time. The normalized protein spot quantity was obtained by dividing the quantity of a spot with the total spot quantities over the whole gel. Only those spots presented in all of the gels used in our experiment were used in the statistical analysis. Owing to our experimental design, which has two biological replications and three subsampling replications nested into each biological replication, the ANOVA of nested design was used to detect protein spots showing statistical difference in abundance between treatments. Thus, the protein spots described in this study showed statistical significance and reproducibility across experiments.

Protein digestion

Using the ProteomeWork gel cutter (Bio-Rad), protein spots were excised from the Sypro-Ruby- (Bio-Rad) stained preparative gels in which 300 μg of proteins of inoculated LSBR-5 were loaded. Six preparative gels were used to obtain sufficient amounts of protein for identification. The excised protein spots were digested with trypsin using the MassPREP station (Waters). The excised spots were de-stained with 50 μL of 50 mM ammonium bicarbonate and 50 μL of 50% acetonitrile, washed once with 50 μL of 100 mM ammonium bicarbonate, and then dehydrated with 50 μL of acetonitrile. The in-gel protein was reduced and alkylated by incubation for 30 min at 56 °C with 10 mM dithioreitol/100 mM ammonium bicarbonate. This reducing solution was then replaced with 55 mM iodoacetamide/100 mM ammonium bicarbonate followed by incubation of protein for 30 min at room temperature. Digestion was carried out with 6 ng/μL trypsin in 25 μL of 50 mM ammonium bicarbonate for 5 h at 37 °C. The digested protein was extracted twice, first with 30 μL of 1% formic acid followed by 12 μL of 1% formic acid/50% acetonitrile.

ESI-Q-TOF MS analysis

The digested protein samples were introduced into a tandem mass spectrometer, Q-TOF Micro (Waters) equipped with a CapLC system (Waters) using the PepMap C18 analytical column (LC Packings, Amsterdam, The Netherlands) at a flow rate of 8 μL/min. The linear gradient was initiated from 95% A (H_2O/0.05% HCOOH) to 65% B (acetonitrile/0.05% HCOOH) in 36 min followed by a linear gradient to 90% B in 2 min. MS/MS spectra were acquired by MassLynx software (Waters). The Q-TOF Micro's internal parameters were set with the electrospray capillary voltage at 3.0 kV, the cone voltage at 30 V and the source temperature at 80 °C. The MS survey scan was *m*/*z* 300–1600 with a scan time of 0.9 s and an interscan time of 0.1 s. The minimum threshold intensity of a peak was 10 counts.

Database searching with MS/MS spectra

MS/MS spectra were used in protein identification using the Mascot MS/MS Ion Search Engine (http://www.matrixscience.com/search_form_select.html). The NCBI rice (*Oryza sativa*) non-redundant protein database was searched first, then the Viridiplantae database was searched if no significantly matched proteins were found or the matched rice proteins were classified as unnamed, unknown or hypothetical. The following search criteria were used: one missed cleavage, variable modifications of carbamidomethyl cystein and oxidation of methionine, ± 2.0 Da peptide tolerance, and ± 0.8 Da MS/MS tolerance.

Location of genetic markers and induced proteins associated with sheath blight resistance on composite genetic/physical map

QTL regions previously identified for sheath blight resistance among F_4 families (Li *et al.*, 1995) and recombinant inbred lines (Pinson *et al.*, 2005) were placed on a composite genetic/physical map and compared with markers selected by discriminant analysis/multiple regression (Pinson, Oard and Capdevielle, unpublished data) and proteins identified in this study (Fig. 4).

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