Plant Omics Journal POJ

POJ 5(6):567-575 (2012) ISSN:1836-3644

Comparative secretome analysis of differentially-induced proteins in rice lesion mimic mutant spotted leaf 11 (*spl*11)

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Abstract

Rice spotted leaf 11 (*spl*11) mutant produces lesions caused by spontaneous cell death without environmental stresses at the three- to four-leaf stages. However, the differential regulation of secreted proteins during cell death process has not yet been explored. Proteins secreted from spotted leaves of the *spl*11 mutant plants and normal leaves of the wild type were extracted using a calcium chloride extraction method, followed by phenol extraction. Comparative secretome analysis using 2-DGE coupled with MALDI-TOF-MS was then applied to these secreted proteins and 28 protein spots were found to be differentially regulated in *spl*11 compared to wild type rice. Two of them were highly accumulated in the wild type, three were highly expressed in *spl*11, and 22 were only detected in *spl*11 mutant. MALDI-TOF MS analysis of 19 spots revealed that they were related to multiple molecular functions, such as photosynthesis (oxygen-evolving enhancer protein, ribulose bisphosphate carboxylase), plant defense (thaumatin-like protein, beta-1,3-glucanase), ROS detoxifying (Cu/Zn-superoxide dismutase, peroxidase), and glycolysis (glyceraldehyde-3-phosphate dehydrogenase). Overall, the results presented here represent the first report of a secretome analysis of *spl*11 mutants as a model system and demonstrate that spontaneous cell death progress was tightly associated with host defense related protein expression and secretion, which was similar to auto-activation of the host defense process.

Keywords: *Oryza sativa*, lesion mimic mutant, *spl*11, secreted protein, proteomics.
Abbreviations: *blm* - blast lesion mimic; CBB - Coomassie Brilliant Blue; DTT - dithiothreitol; EMS - ethyl methanesulfonate; GAPDH - glyceraldehyde-3-phophate dehydrogenase; G6PDH - glucose 6-phosphate dehydrogenase; HR - hypersensitive response; OEE1 - oxygen-evolving enhancer protein; PR - pathogen related; ROS - reactive oxygen species; SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis; *spl* - spotted leaf; SOD - superoxide dismutase; TFA - trifluoroacetic acid; TLP - thaumatin-like protein; XIP - xylanase inhibitor protein; 2-DGE - two - dimensional electrophoresis.

Introduction

Hypersensitive response (HR) is a typical defense mechanism to block infection by bacterial and fungal pathogens that results in the protection of the host (Dangle and Jones 2001). HR in plants is always accompanied by accumulation of reactive oxygen species (ROS) and expression of pathogen related (PR) proteins (Dixon et al., 1994). Previous studies have shown that the HR phenotype occasionally occurs in mutants without infection, not only in rice (Kiyosawa 1970, Marchetti et al., 1983), but also other cereals (Johal et al., 1995, Kjaer et al., 1990, Morris et al., 1998). Mutants with spontaneous cell death phenotypes have been isolated through rice mutant screening, and named spotted leaf (*spl*) mutants by different research groups (Takahashi et al., 1999, Yin et al., 2000). To date, at least 27 lesion mimic mutants have been identified from *spl* series, seven of which are dominant (Wu et al., 2008). Three genes, *spl*7, *spl*11, and *spl*18, encoding heat shock transcription factor, E3 ubiquitination ligase, and acyltransferase, respectively, have been characterized (Mori et al., 2007, Yamanouchi et al., 2002, Zeng et al., 2004). These mutants show self-activation of defense related proteins and non-race-specific resistance to fungal and bacterial pathogens, and have served as a good material for study of the rice defense mechanism (Takahashi et al., 1999, Yin et al., 2000, Wu et al., 2008). Therefore, investigation of *spl* mutants using genetics, proteomics or other molecular biological methods have been conducted to provide new insight into early signaling of rice defense regulation. With the improvement of identification methods, proteomics is a powerful tool for large scale screening and specific identification of differentially expressed proteins by separating proteins based on p*I* and molecular weight. Several proteomic approaches have been used to investigate *spl* mutants to understand initiation of defense signaling in rice (Takahashi et al., 1999). CDR1 and CDR2, which were characterized from rice lesion-mimic mutants, showed resistance against rice blast fungus (Takahashi et al., 1999). Both genes could suppress protein phosphorylation of NAPDH oxidase, and lead to blocking of oxidative burst against pathogen attacks (Takahashi et al., 1999), suggesting that

CDR1 and CDR2 negatively regulate the phosphorylation of proteins, or chaperone protein prohibitin (OsPHB1), or that they are involved in defense response or programmed cell death associated with NADPH oxidase (Takahishi et al., 2003). Later, a proteomic analysis of *cdr2* was carried out using total protein extraction, and 37 proteins were found to be up- or down-regulated in the *cdr* mutant (Tsunezuka et al., 2005). The proteins involved in the metabolic processes were major identified proteins when compared to host defense related proteins, indicating that programmed cell death in the *cdr2* mutant is associated with active metabolic changes (Tsunezuka et al., 2005). Proteomic analysis of the rice blast lesion mimic (*blm*) mutant, revealed 50 spots that were differentially expressed, and 26 non-redundant proteins were identified from *blm* rice mutant leaves in the early seedling stages (two-week-old) (Jung et al., 2006). Among the identified proteins, OsPR5 and OsPR10 were up-regulated at both the protein and RNA levels (Jung et al., 2006). Proteomic analysis of *spl*6 mutant showed differential regulation of 159 protein spots when compared to the wild type. However, only 25 spots encoding 13 unique proteins were identified. The identified proteins were found to be related to metabolism, photosynthesis, energy, and stress resistance (Kang et al., 2007). Among them, anti-oxidative burst related proteins, disulfide isomerase, and thioredoxin peroxidase, were not detected in the *spl*6 mutant, suggesting that cell death and lesion may be caused by oxidative burst in plants (Kang et al., 2007). Recently, a proteomic investigation of *spl*1 was reported, and 18 protein spots were identified from 2-DGE including a cell death related protein PBZ1 (Kim et al., 2008). GFP fusion of PBZ1 protein showed a co-localization of programmed cell death pattern in rice, indicating that PBZ1 may be a molecular marker of rice defense and cell death. These proteomic analyses indicated that investigation of those lesion mimic mutants can provide new information useful to characterization of programmed cell death and defense related proteins. Previously, a spotted leaf11 mutant (*spl*11) was generated from the IR68 line by ethyl methanesulfonate (EMS) mutagenesis (Singh et al., 1995) and found to have high resistance to both *Magnaporthe oryzae* and *Xanthomonas oryzae* infection (Yin et al., 2000). The *spl*11 gene was characterized by genetic mapping, which indicated that the cell death in *spl*11 was caused by a single mutation of the SPL11 protein (Zeng et al., 2002, Zeng et al., 2004). SPL11 is a protein containing a U-box-domain that shows strong E3 ubiquitin ligase activity, suggesting that the regulation of ubiquitin mediated protein degradation is involved in rice defense and programmed cell death. However, no further studies have been conducted to understand downstream protein regulations in *spl*11 mutants to date. Therefore, in this study, we employed proteomics to analyze differentially regulated secretory proteins in *spl*11 during cell death. Previous studies have indicated that protein secretions are important in the interaction between hosts and pathogens (Büttner and He 2009, Wang and Dong 2011), and it is known that proteins from the host side are essential to activation of host immune responses (Kwon et al., 2008). Therefore, rice secretome analysis in response to environmental stresses will help us understand how plants adapt to stresses. To accomplish this, we first developed an efficient method for the extraction of secreted proteins from rice leaves, and then applied a proteomic approach to analysis of those proteins. Differentially expressed secretory proteins in the *spl*11 mutant were subsequently detected by 2-DGE and identified by MALDI-TOF MS, which revealed that they were related to photosynthesis, ROS detoxification and host defense mechanisms. Overall, the results presented here provide an in-depth understanding of the molecular mechanism of lesion formation and defense activation.

Results

Phenotype analysis of spl11 mutant

The rice *spl*11 mutant was previously shown to display a spontaneous cell death phenotype (Singh et al., 1995). *Spl*11 encodes a U-box/armadillo repeat protein that functions as a negative regulator of rice defense (Yin et al., 2000). The investigation of defense signaling regulation is important to understanding rice defense mechanisms. Therefore, we analyzed the cell death phenotype in *spl*11 mutant leaves. Visible cell death did not occur in the germination and early developmental stages (data not shown). A few lesions were initiated at the 2-3 leaf stages, and significant cell death was detected at the four-leaf-stages (Fig. 1*A*). In mature plants, serious cell death lesions appeared in most leaf areas and stems (Fig. 1*B*). Therefore, we selected the three- to four-leaf-stage plant for our proteomic analysis, which was the time point of initiation of cell death and defense in plants.

Pure extraction of secreted proteins from spl11 leaves

Recent reports have suggested that the apoplastic region, which contains enriched secreted protein, is the first and direct point of the host pathogen interaction (Kankanala et al., 2007), and that proteins secreted from rice are essential for plant innate immunity (Kim et al., 2009). Therefore, investigation of differential regulation of secreted proteins from wild type and *spl*11 mutants are important to understanding the rice innate immunity mechanism. To analyze the proteins secreted from rice leaves, a clear and efficient extraction method is needed. Therefore, we developed a novel method, known as the $CaCl_2$ extraction method, to improve the extraction of secretory proteins from rice leaves. Briefly, leaves at the 3 or 4-leaf-stage were cut into pieces (4-5 cm) and then incubated on ice using $CaCl_2$ extraction buffer to extract apoplastic accumulated proteins (Fig. 2). The incubation process increased the efficiency of secreted protein extraction, reduced wounding caused contamination, and therefore strongly increased the extraction efficiency. We also estimated intracellular marker enzyme activity, namely, glucose 6-phosphate dehydrogenase (G6PDH). Little G6PDH activity was detected in apoplastic proteins extracted from wild type and *spl*11 mutants, indicating that the secretome prepared from rice leaves was essentially free from contamination of non-secreted proteins (data not shown). The secreted proteins were then extracted with phenol and precipitated and separated by 2-DGE with a p*I* of 4~7 and molecular mass of 11~95 kDa (Fig. 2).

Differential expression of secreted proteins in spl11

When compared with the 2-DGE map of the wild type, several protein spots were commonly detected in *spl*11 mutant, suggesting they may be constitutively expressed in the apoplastic region, and therefore related to development of other molecular functions (Fig. 3). Moreover, most protein spots showed a high accumulation in the *spl*11 leaf, suggesting they are related to with cell death and defense mechanisms. Overall, 28 differentially regulated spots ($p < 0.05$) were selected from the 2-DGE maps (Fig. 3). A close view of each of these spots was generated (Fig. 4*A*) and their intensity was measured (Fig. 4*B*). Among the selected spots, only two (spots 12 and 13) were highly accumulated in the wild type, while 26 spots (spots 15,

Table 1. Identification of differentially induced apoplastic secreted proteins in *spl11* mutant using MALDI-TOF MS.

No.	Protein name	Locus	Score	Search engines	MP	SC (%)	Ex Mr	Th Mr	Ex *pI*	Th *pI*	SignalP	SecretomeP	Function
2	Thaumatin-like protein	Os12g43380	4079	Prospector	3	21	27.4	18.7	4.71	5.1	Y		Defense
3	β-1,3-glucanase	Os01g71340	422	Prospector	4	15.9	22.2	28.2	4.6	4.7	Y		Defense
6	Cu/Zn-superoxide dismutase	Os08g44770	67	Mascot	4	31	15.9	20519	5.63	5.79	Y		ROS detoxify
7	Thaumatin-like protein	Os12g43380	2191	Prospector	4	24.3	14.8	17.9	5.81	5.1	Y		Defense
9	Oxygen-evolving enhancer protein 1	Os01g31690	95	Mascot	6	28	30.7	34.8	5.38	6.1		Y	Photosynthesis
10	Oxygen-evolving enhancer protein 2	Os07g04840	103	Mascot	7	35	24.8	26.9	5.75	8.66		Y	Photosynthesis
11	Peroxidase	Os07g48020	86	Mascot	7	29	32.5	32.8	5.86	5.77	Y		ROS detoxify
12	Ribulose bisphosphate carboxylase	Os10g21268	68	Mascot	6	14	54.1	52.7	6.17	6.23		Y	Photosynthesis
13	Ribulose bisphosphate carboxylase	Os10g21268	68	Mascot	6	14	54.9	52.7	6.11	6.23		Y	Photosynthesis
15	Glyceraldehyde-3-phosphate dehydrogenase	Os08g03290	105	Mascot	8	27	39.9	36.3	6.58	6.61		Y	Glycolysis
16	Oxygen-evolving enhancer protein 2	Os07g04840	103	Mascot	7	35	24.9	26.9	5.81	8.66		Y	Photosynthesis
17	Oxygen-evolving enhancer protein 2	Os07g04840	103	Mascot	7	35	23.7	26.9	5.92	8.66		Y	Photosynthesis
20	Ribonuclease	Os08g33710	67	Mascot	5	27	21.8	23.5	4.81	4.95	Y		RNA catabolic process
21	Secretory protein	Os10g34930	81	Mascot	5	20	22.9	24.6	5.37	5.5	Y		Transport
22	Oxygen-evolving enhancer protein 2	Os07g04840	103	Mascot	7	35	24.2	26.9	5.67	8.66		Y	Photosynthesis
23	Glyceraldehyde-3-phosphate dehydrogenase	Os04g40950	87	Mascot	7	33	38.8	36.7	6.44	6.34			Glycolysis
24	Glyceraldehyde-3-phosphate dehydrogenase	Os04g40950	87	Mascot	7	33	34.5	36.7	6.44	6.34			Glycolysis
25	Xylanase inhibitor protein 1	Os06g25010	65	Mascot	5	24	30.7	27.4	6.47	6.35	Y		Defense
26	Peroxidase	Os07g48020	71	Mascot	6	23	33.6	37.3	5.63	5.8	Y		ROS detoxify

No., Spot number; MP, matched peptides; SC, sequence coverage; Ex Mr, experimental molecular mass (kDa); Th Mr, theoretical molecular mass (kDa); Ex p*I*, experimental isoelectric point; Th pI theoretical isoelectric point; SecretomeP, secretion predicted by the SecretomeP program.

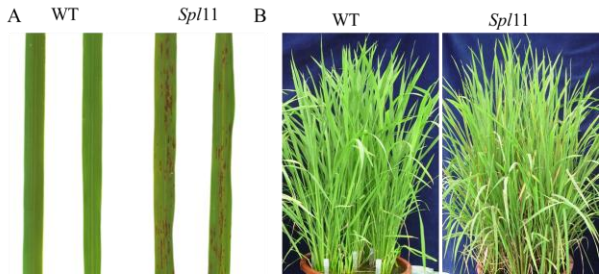


Fig 1. Phenotype analysis of wild type (WT) and *spl*11 mutant leaves. (A) Lesion symptoms in leaves of the WT and *spl*11 mutant. (B) Lesion symptoms in of the entire *spl*11 mutant plant.

17, and 20) were highly accumulated in *spl*11, including 23 *spl*11 specific spots (Fig. 4).

Identification of proteins secreted from spl11

To determine which proteins were expressed during this early defense and cell death mechanism, all spots from 2-DGE maps were digested and subjected to MALDI-TOF-MS identification. Seven spots (spots 1, 4, 5, 8, 14, 18, and 19) did not match any proteins in the Prospector or Mascot database. Nineteen spots were identified and found to be encoded by 12 unique proteins (Table 1). The putative molecular functions of these proteins were then predicted using the UniProt database (http://www.uniprot.org/). Four proteins, including oxygen-evolving enhancer protein 1 (spot 9), oxygen-evolving enhancer protein 2 (spots 10, 16, 17, and 22), and ribulose bisphosphate carboxylase (spots 12 and 13), were found to be related to photoinhibition/photosynthesis. Spots 12, 13 were decreased in *spl*11 when compared to the wild type, while the others were increased (Fig. 4*B*). Three proteins, thaumatin-like protein (TLP, spots 2, 7), β-1,3-glucanase (spot 3), and xylanase inhibitor protein 1 (XIP1, spot 25), were related to host defense mechanisms. All defense related proteins were highly expressed in *spl*11, but not detected in the wild type (Fig. 4*B*). Two of each proteins found to be related to oxidative stress, Cu/Zn-SOD (spot 6) and peroxidase (spots 11 and 26), were only detected in *spl*11, while three spots encoding two different glyceraldehyde-3-phophate dehydrogenases (GAPDH) (spots 15, 23, and 24) which is involved in glycolysis, were highly (spot 15) or specifically (spots 23 and 24) accumulated in *spl*11.

In silico confirmation using SignalP 4.0 (www.cbs.dtu.dk /services/SignalP/) and secretomeP (www.cbs.dtu.dk/ services/SecretomeP/) was employed to predict protein secretion. Specifically, the SignalP program was used for screening of classical secretion proteins (ER-Golgi mediated) that contain signal peptides and cleavage sites (Nielsen et al., 1997, Petersen et al., 2011), while SecretomeP was used for prediction of non-classical or leaderless secretion proteins (Bendtsen et al., 2004). Among them, eight proteins (10 spots) contained N-terminal signal sequence, and four (8 spots) were predicted to be secreted proteins by SecretomeP (Table 1). These results suggest that both the classical ER-Golgi mediated secretion pathway and non-classical or leaderless secretion pathway are important for protein secretion during rice immune response. However, one spot (GAPDH, spot 15) could not be predicted by either program, suggesting that other secretion methods are needed to identify protein outer membrane localization processes such as exocytosis.

Discussion

In this study, we applied comparative proteomic analysis to understand the differential expression of secreted proteins in the apoplastic region from wild type and lesion mimic mutant *spl*11 at the stage of cell death initiation. The apoplastic region is a narrow space between the rice plasma membrane and the fungal invasive hyphae membrane that is very important for host defense and pathogen infection (Kankanala et al., 2007). Proteins secreted from both the host and pathogen sides were accumulated in this region, indicating that the protein-protein interactions between host and pathogen occur first and directly in this area. Therefore, understanding protein accumulation in the apoplastic region will provide insight into the host defense mechanism. Previously, a gravity extraction method employing centrifugation was applied to extract secreted proteins from leaves (Jung et al., 2008). This extraction method enabled a high purity of secreted protein extraction by preventing intracellular contaminations. However, our $CaCl_2$ extraction method was highly efficient at extracting proteins from wounded or cell death tissues. Thus, only a few protein spots were detected in the extracts secreted from control leaves when compared to those extracted from *spl*11 leaves. Therefore, this method could be used for the detection of proteins from wounded or lesion tissues, especially pathogen infected tissues, and provide a new and useful tool for investigation of protein secretion and interaction between plants and pathogens. Based on our proteomic analysis of secreted proteins extracted from the *spl*11 apoplastic region, 28 differentially expressed proteins were detected on 2-DGE maps (Fig. 3), and most of those proteins were up-regulated in *spl*11 (Fig. 4*B*), indicating that they were associated with the cell death process. As previously reported, *spl*11 showed resistance to pathogen attack; therefore, we speculate that those secreted proteins are related to the anti-pathogen process. Photosynthesis related proteins were found to be the most common and highly conserved protein groups upon secretome analysis. The oxygen-evolving enhancer protein 1 (OEE1) and OEE2 are components of photosynthesis complex II related to oxygen generation in plants (Thornton et al., 2004). OEE1 and OEE2 have been reported to be involved in an inevitable consequence of oxygenic photosynthesis known as photoinhibition (Raven 2011). Expression of these photoinhibition enzymes results in suppression of the photosynthesis ratio and protection of the cell against ROS induced damage during photosynthesis (Raven 2011). Rubisco bisphosphate carboxylase (spots 12 and 13) is one of many enzymes involved in the Calvin cycle, which is related to carbon fixation during photosynthesis (Spreitzer and Salvucci 2002). Based on our proteomic analysis, both OEE1 (spot 9) and OEE2 (spots 10, 16, 17, and 22) were significantly increased in *spl*11, but the ribulose bisphosphate carboxylase (spots 12 and 13) was decreased when compared to wild type plants. These data suggest that the photosynthesis process was strongly suppressed in *spl*11 during cell death. ROS including superoxide and hydrogen peroxide are generated as secondary messengers in host defense mechanisms (Orozco-Cardenas et al., 2001), and rapidly accumulate in the apoplastic region in response to pathogen attack (O'Brien et al., 2012). Two ROS detoxify related proteins, Cu/Zn-SOD (spot 6) and peroxidase (spots 11 and 26) were identified from secretome analysis of the *spl*11 mutant (Table 1). Cu/Zn-SOD can catalyze superoxide to hydrogen peroxide, and peroxidase utilizes hydrogen peroxide as a substrate to generate water and oxygen. Previous research showed that SOD proteins were increased in the lesion mimic mutant during lesion formation (Jung et al., 2006), and peroxidases were induced in response to rice blast fungus infection (Sasaki et al., 2007). The accumulation of such ROS detoxifying proteins could help plants maintain ROS balances and suppress additional damage caused by ROS. High accumulation of ROS signals also leads to the activation of defense related proteins. Three rice defense related proteins were identified in our study, TLP (spots 2 and 7), β-1,3-glucanase (spot 3), and xylanase inhibitor protein 1 (spot 25) (Table 1). TLP belongs to the pathogenesis-related group 5 (PR-5) family, and has been found to be expressed during antifungal activity via interference with the integrity of the fungal cell membrane (Tobias et al., 2007). TLP has been reported to be highly expressed in response to fungal (*Magnaporthe oryzae*) or bacterial (*Xanthomonas oryzae*) infection (Kim et al., 2004, Mahmood et al., 2006). Over-expression of rice TLP can enhance resistance to *Fusarium oxysporum* infection in banana plants (Mahdavi et al., 2012). β-1,3-glucanase, which is a hydrolytic enzyme that can hydrolyze the β-1,3-linked glucans on fungal cell walls and lead to activation of host defense (Kim et al., 1997, Keen et al., 1983),

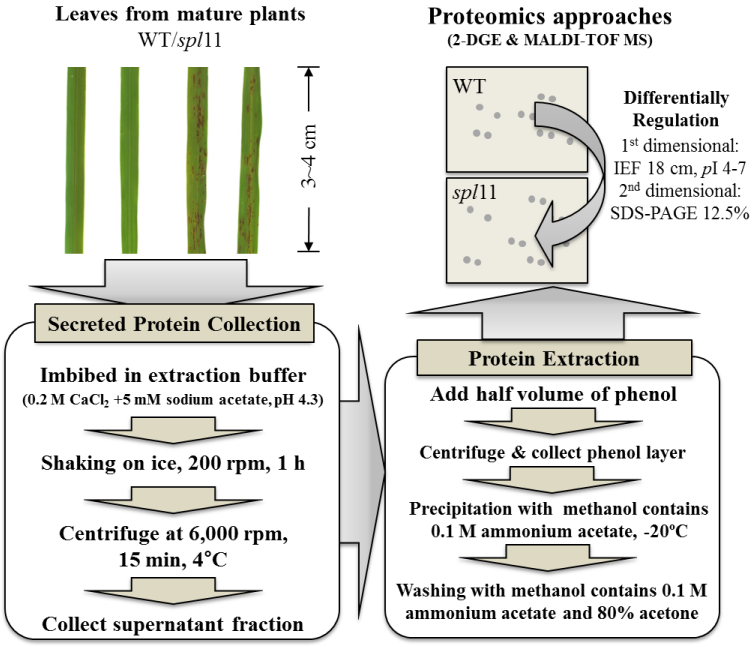
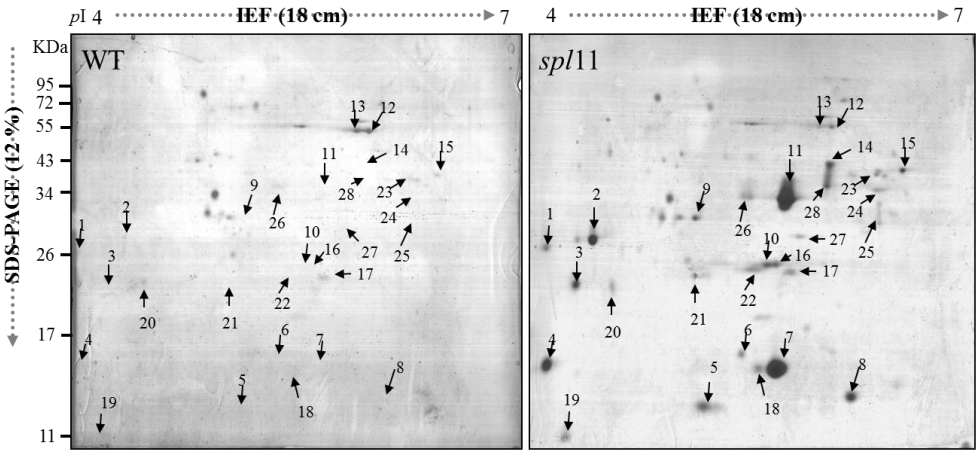


Fig 2. Experimental procedure for extraction of secreted proteins. Secreted proteins from WT and *spl*11 mutants were extracted using $CaCl_2$ extraction buffer after incubation on ice (Secretion Protein Collection). Total proteins were extracted using phenol and precipitated by methanol containing ammonium acetate. Extracted proteins were separated on 18 cm IEF gel (*pI* 4-7), followed by 12.5% SDS-PAGE. Differentially expressed proteins were then identified by MALDI-TOF MS.



Wang et al Figure 3 blade of WT and *spl*11 mutant. Secretory proteins extracted from WT and *spl*11 were separated on 2-DGE and stained with Coomassie Brilliant Blue. Differentially expressed proteins were numbered and were indicated by arrows.

was previously classified as a PR-2 family member and shown to be, related to pathogen attack in rice, especially that by rice blast fungus (Kim et al., 2004). The expression of twenty-seven rice β-1,3-glucanases in different tissues and in response to biotic and abiotic stresses was analyzed by Northern blot analysis, which revealed that most β-1,3-glucanases were activated in response to fungal attack (Hwang et al., 2007). Additionally, xylanase inhibitor proteins were activated in response to fungal infection in wheat, and found to exert specific and competitive inhibition of fungal xylanases, but have no effect on bacterial enzymes (Flatman et al., 2002, Juge et al., 2004, Igawa et al., 2004). In rice, three xylanase inhibitor proteins were previously analyzed and fond to show differential organ-specific expression. Wounding stress and JA treatment have been shown to strongly activate the expression of these xylanase inhibitor proteins, suggesting that xylanase inhibitor proteins were involved in rice defense mechanism (Tokunaga and Esaka 2007). These three rice defense related proteins were found to be secreted in the *spl*11 mutant during cell death, and their accumulation may act as the first line of defense during pathogen infection. TLP and β-1,3-glucanases directly attack pathogens to suppress their infection by interfering with or degrading their cell wall structure. Xylanase inhibitor protein suppresses fungal xylanase activity to protect the host cell wall, and leads to blockage against host infection. The glycolysis related protein, GAPDH, which is involved in energy (ATP) generation, was identified during our secretome analysis. Three spots (spots 15, 23, and 24) that encoded GAPDH were more highly accumulated in the *spl*11 than the wild type. The secretion of spot 15 was mediated by a non-classical secretion

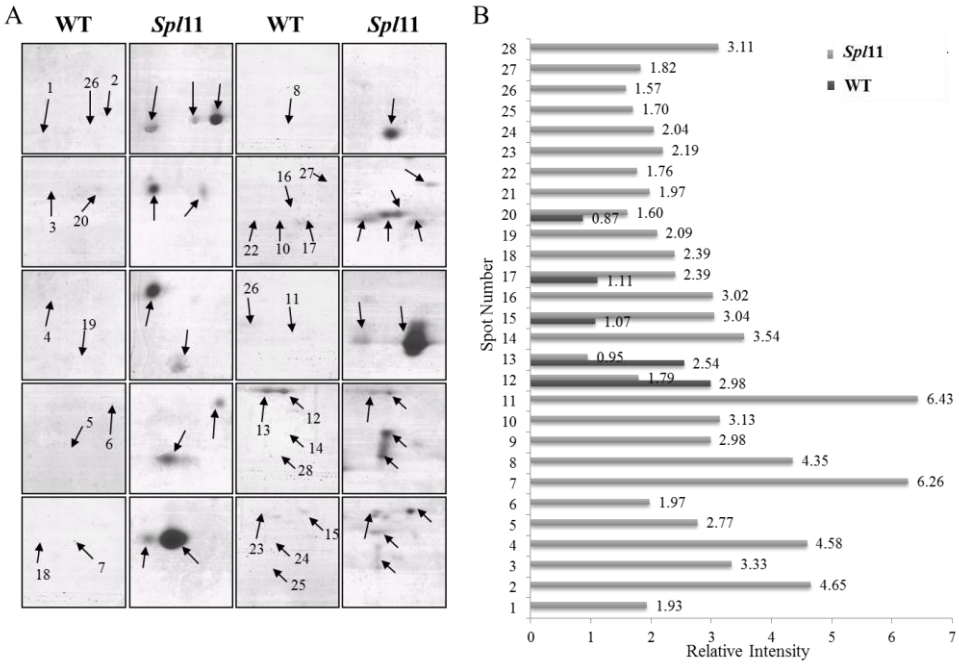


Fig 4. Expression profiles of protein spots in WT and *spl*11 mutant. (A) A close-up view of differentially expressed protein spots was generated. (B) The relative intensities of each spot were measured using ImageMaster software, and the bar graph was generated based on the average intensity of three gel replicates.

pathway, and spots 23 and 24 were mediated by unknown pathways. In plants, GAPDH was involved in abiotic and biotic stress adaptations, including anaerobic stress (Russell and Sachs 1989, Russell et al., 1990), heat (Yang et al., 1993), salt (Zhang et al., 2011), ROS (Hancock et al., 2005), elicitor (Laxalt et al., 1996), *spl*6 mutant (Kang et al., 2007), and brown plant hopper (Wei et al., 2009). Therefore, the accumulation of GAPDH during spontaneous cell death may be activated by stress signaling, especially in response to ROS, and related to stress adaption processes in rice. However, further studies are required to confirm this. A secretomic analysis using the *spl*11 mutant was first carried out in this study, and the results revealed differential regulation and secretion of proteins related to photosynthesis, plant defense, and the ROS detoxifying process. Our findings suggest that loss of function of SPL11 in rice could suppress plant photosynthesis, activate host immunity, and generate a high level of ROS in apoplastic regions. ROS function as a secondary signaling pathway to induce secretion of ROS detoxifying proteins and defense-related proteins, and finally lead to spontaneous cell death in leaves. Additionally, the secretion of ROS detoxifying and defense related proteins increases plant non-host specific resistance. When compared with previous proteomic studies, differentially regulated proteins in *blm* are most similar to those in *spl*11. OEE, GAPDH, TLP, and SOD were all up-regulated in *blm* and *spl*11, suggesting that the molecular mechanism of cell death and defense regulation in *blm* and *spl*11 may be similar (Jung et al., 2006). Rubisco protein, GAPDH, and SOD were also detected in *cdr2* (Tsunezuka et al., 2005), and GAPDH and TLP were highly expressed in *spl*6 (Kang et al., 2007). ROS detoxifying related proteins including SOD, ascorbate peroxidase, catalase, and glutathione S-transferase are commonly detected in *spl* mutants (Jung et al., 2006, Kang et al., 2007, Kim et al., 2008, Tsunezuka et al., 2005). Taken together, these results suggest that the cell death activation mechanism in apoplastic region is similar, at least in part, to those in lesion mimic mutants, and that ROS play an important role in the signaling.

Materials and methods

Plant materials and protein extraction

Rice seeds (*Oryzae sativa*) and *spl*11 seeds were imbibed in distilled water at 4°C for three days, and then planted in field soil in a green house. Leaves of plants at the three- to four-leaf-stage were used for protein extraction. Proteins secreted from leaves were extracted by the calcium extraction method. Briefly, fifty grams of rice leaves were cut with scissors to an average length of 4-5 cm, imbibed with 200 ml calcium extraction buffer (200 mM $CaCl_2$, 5 mM Na-acetate, pH 4.3.), and then shaken on ice for 1 h. The extraction buffer was then collected by vacuum filtration and centrifugation at 3,000 rpm for 15 min at 4°C, after which half the volume of water saturated phenol was added to the extraction buffer. The samples were subsequently shaken for 10 min on ice, after which they were centrifuged at 3000 rpm for 10 min. The phenol layer was then collected and precipitated with methanol containing 0.1 M ammonium acetate for 2 h at -20°C. Next, the sample were centrifuged at 3000 rpm for 10 min, after which the pellet was washed with methanol containing 0.1 M ammonium acetate four times, followed by two washes with 80% acetone. Finally, the samples were stored in 80% acetone at -20°C until used. The protein content in the finally prepared sample was measured through the method described by Kim et al. (2008).

DGE, image analysis, and MALDI-TOF-MS

The 2-DGE, image analysis of 2-D gels, and trypsin digestion of 2-D protein spots were performed essentially as described by Kim et al. (2004). Briefly, proteins extracted from the same amount of leaf samples were mixed with rehydration buffer [8 M (w/v) urea, 2% (w/v) CHAPS, 0.002% (w/v) Bromophenol G, 20 mM dithiothreitol (DTT), 0.5% (v/v) pharmalyte (pH 5–8)] and loaded onto pre-cast IPG strips (18 cm, *p*I 4.0-7.0) using an IPGphor 3 platform (GE healthcare, Sweden) for 12 h. The samples were then focused at 50 V for 8 h, 100 V for 1 h,

500 V for 1 h, 1000 V for 1 h, 2000 V for 1 h, 4000 V for 2 h, 8000 V for 5 h, 8000 V for 3 h, and 20 V for 2 h. Next, the IPG strips were then separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the secondary dimensional analysis, after which they were stained with Coomassie Brilliant Blue G-250 (CBB). The colloidal CBB stained 2-D gels were then scanned (300 dpi, 16-bit gray scale pixel depth, TIFF file) for image analysis using the ImageMaster 2D Platinum imaging software ver. 6.0 (GE Healthcare, Sweden). Finally, the intensities of individual spots were normalized based on the total density of all spots detected in each gel and compared between gels.

In-gel digestion and mass spectrometry

Protein spots were carefully excised from CBB-stained gels, digested with trypsin, and extracted peptides according to the method of described by Kim et al., (2008). Briefly, Protein spots excised from CBB-stained gels were reduced with 10 mM DTT solution in 0.1 M NH_4HCO_3 at 55 °C for 45 min, and replaced with 55 mM iodoacetamde in 0.1 M NH_4HCO_3 for 45 min in the dark.. The gel was washed with 50% ACN in 0.1 M NH_4HCO_3 and dried. The dried gel was digested with trypsin enzyme in 10 μL digestion buffer (25 mM NH_4HCO_3, 0.1% n-octyl glucoside (w/v), 12.5 ng/μL trypsin) at 37 °C overnight. The digested peptide was subsequently dissolved in H_2O: acetonitrile:trifluoroacetic acid (TFA)=93:5:2 and sonicated for 5 min. A 2 μl mixture of matrix solution [40 mg/ml a-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, St. Louis, MO, USA) in acetone and 20 mg^{-1} mL nitrocellulose in acetone:nitrocellulose:isopropanol= 2:1:1 was then added to 2 μl of peptide sample solution. Next, 1 μl of solution mixture was immediately spotted onto a matrix-assisted laser desorption/ionization plate, and allowed to stand for 5 min at RT. The plate was then washed with 0.1% TFA and analyzed by Voyager-DE STR MALDI-TOF-MS (PerSeptive Biosystems, Framingham, MA, USA). Parent ion masses were subsequently measured in reflection/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 76.000%, a guide wire voltage of 0.010%, and a delay time of 150 ns. Des-Arg1-bradykinin (m/z 904.4681) and angiotensin 1 (m/z 1296.6853) were used as a two-point internal standard for calibration. Peptides were selected in the mass range of 600-3,000 Da. For data processing, the Moverz software package (http://bioinformatics.genomicsolutions.com/moverz.html) was used. Database searches were performed using Protein Prospector (http://prospector.ucsf.edu) and Mascot (http://www.matrixscience.com) websites against *O. sativa*. Spectra matching proteins are provided (Supplementary data).

Conclusion

This study established an efficient extraction method to isolate and to investigate apoplastic proteins secreted from rice leaves. For example, we also carried out secretome analysis to understand cell death in *spl*11mutant as a model system. From secretome analysis combined with $CaCl_2$ extraction method, twenty five spots were highly expressed or only detected in *spl*11 mutant, compared to wild-type rice by using 2-DGE and 19 proteins were identified by MADLI-TOF MS. The differentially expressed proteins in the *spl*11 mutant were mainly involved in photosynthesis, plant defense, ROS detoxification, and glycolysis. The study of secretory proteins from *spl*11 can provide important clues for understanding the complex cell death mechanisms in apoplastic region during plant and microbe interaction. The established extraction method for secretome will also serve as a resource to help further elucidation of the mechanisms involved in rice self-defense response.

Acknowledgements

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0004087), by a grant from the Next-Generation BioGreen 21 Program (Plant Molecular Breeding Center, PJ008021) and by scholarships from the Brain Korea 21 Program (D.Y. Lee).

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