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Proteomic analysis of bacterial blight defence signalling pathway using transgenic rice overexpressing thaumatin-like protein

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Abstract

Rice overexpressed thaumatin-like protein gene and the proteins from the leaf blades of 2-week-old transgenic rice seedlings were fractionated into cytosolic and membrane fractions, and separated by two-dimensional polyacrylamide gel electrophoresis and stained with Commassie brilliant blue. Among of 440 detected proteins, 5 proteins were up-regulated and 5 proteins were down-regulated by the overexpression of thaumatin-like protein. In the sense thaumatin-like protein transgenic rice and/or in rice inoculated with *Xanthomonas oryzae* pv. *oryzae* (Xo7435), 2-cys peroxiredoxin, thaumatin-like protein and glycine cleavage H protein were up-regulated, while oxygen evolving complex protein 2 was down-regulated. These results suggest that thaumatin-like protein-mediated disease resistance of rice against bacterial blight disease is the results of changes in proteins related to oxidative stress and energy metabolism in addition to changes in proteins related to defence.

Additional key words: Oryza sativa, oxygen evolving complex, *Xanthomonas oryzae* pv. *oryzae.*

Introduction

The degree of resistance to bacterial blight of rice has been variable among different rice cultivars (Ezuka and Kaku 2000). The occurrence of disease or induction of resistance depends on the resistance genes of the plant and their cognate avirulence genes of the pathogen, a phenomenon known as gene-for-gene interaction (Shen and Ronald 2002). The interaction between *Xanthomonas oryzae* pv. *oryzae* and rice was either compatible or incompatible, resulting into either disease occurrence or resistance response (Yoshimura *et al.* 1960).

Rice exhibited a variety of responses during infection by pathogens or to abiotic stresses, many of which involved the activation of defence related genes. Activation of these genes brought about physical and biochemical changes, including the accumulation of pathogenesis-related (PR) proteins (Van Loon *et al.* 1994). PR proteins were not only coded by the host plants, but also induced in response to the interaction between host and virus (Antoniw and Pierpoint 1978). Further, manipulation of resistance genes and their signalling pathways by transgenic expression was a promising strategy to improve disease resistance in plants (Van Loon *et al.* 1994). Thus, preparation of disease resistant transgenic rice meant to reduce dependency on disease control by chemicals and to understand the molecular basis of the interaction between plants and pathogens.

Thaumatin-like protein, osmotins or zeamatins have been categorized as PR5 protein (Stintzi *et al.* 1993). These proteins were acidic or basic according to their isolelectric focusing points and the genes for either of the forms have been isolated (Van Kan *et al.* 1989, Nelson *et al.* 1992). Several members of PR5 proteins had differential antifungal activities specific to different fungal species (Vigers *et al.* 1992). PR10 knocked out mutant of *Medicago truncatula* resulted into the increased expression of PR5, thereby increased resistance against *Aphanomyces euteiches* (Colditz *et al.* 2007). Osmotin has been reported to be involved in the defence of potato plant against *Phytophthora infestans* (Takemoto *et al.* 1997, Zhu *et al.* 1996). Further, Rajam *et al.* (2007) reported the development of transgenic tobacco plants with thaumatin gene of *Thaumatococcus daniellii* under the control of strong constitutive promoter. Transgenic plants exhibited enhanced resistance against *Pythium aphanidermatum* and *Rhizoctonia solani* with delayed disease symptoms (Rajam *et al.* 2007).

The over-expression of thaumatin-like protein enhanced the resistance of transgenic rice against sheath

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; 2D-PAGE - two-dimensional polyacrylamide gel electrophoresis; MS - mass spectrometry; NAA - naphthaleneacetic acid; PR - pathogenesis-related; PVDF - polyvinylidine difluoride.
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blight disease caused by *R. solani* (Datta *et al.* 1999). Mahmood *et al.* (2006) has reported the up-regulation of thaumatin-like protein in rice by the inoculation of *X. oryzae* pv. *oryzae* and by the exogenous application of jasmonic acid. Based on these results, it was suggested that manipulation of thaumatin-like proteins would result into change in the resistance against various plant pathogens. Further, these results indicated the importance of thaumatin-like protein in the defence response of rice. To evaluate the resistance conferred by thaumatin-like protein against bacterial blight of rice, thaumatin-like protein gene overexpressed transgenic rice was prepared and analyzed through proteomic approach.

Materials and methods

Preparation of transgenic rice: Dehusked mature seeds of rice (*Oryza sativa* L.) cv. ZTS were sterilized and incubated at 25 °C in growth chamber on the modified callus-induction N6 medium (Chu *et al.* 1975) containing 30 g dm^{-3} sucrose, 0.3 g dm^{-3} casamino acids, 2.8 g dm^{-3} proline, 2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 4 g dm^{-3} *Gelrite*. The induced calli were used for transformation. The open reading frame of thaumatin-like protein gene (accession number AK 058637) was amplified using primer pairs:
5'-CACCATGGCGCCTTCCCTCGCCACCT-3' and
5'-TCATCATGGGCAGAAGACGACCTGGT-3'.
Reaction was performed for 40 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C and extension for 3 min at 72 °C, followed by a final extension of 7 min at 72 °C. The resulting PCR fragment was cloned in pH2GW7 and pH2WG7 binary vectors (*Invitrogen*, Carlsbad, CA, USA) in sense and antisense orientations, respectively and confirmed by sequencing. Sequencing was done using dye-labelled terminations (*PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit*; *Applied Biosystems*, Foster City, CA, USA) and an automated DNA sequencer (*ABI PRISM 310 Genetic Analyzer*; *Applied Biosystems*). DNA sequence was analyzed with *Gentyx-WIN Version 5.1* (*Software Development*, Tokyo, Japan).

The recombinant vectors and corresponding vectors were transformed into *Agrobacterium tumefaciens* strain EHA 105 (Hood *et al.* 1986) and transformed into rice (Toki *et al.* 1997), except EHA105 containing the binary vector was grown for 3 d on the Luria-Bertani (LB) medium (Maniatis *et al.* 1982; pH 7.5) containing 10 g dm^{-3} bacto-tryptone, 5 g dm^{-3} yeast extract, 10 g dm^{-3} NaCl, 15 g dm^{-3} agar, 100 mg dm^{-3} spectinomycin and 50 mg dm^{-3} hygromycin. The bacterial cells were resuspended in medium (Hiei *et al.* 1994) containing 10 mg dm^{-3} acetosyringone. The calli were soaked in this suspension for 2 min and blotted dry, using sterile Kimwipes for removal of excess bacteria. A co-culture was performed on modified N6 medium containing 10 mg dm^{-3} acetosyringone (Hiei *et al.* 1994) for 2 d. After co-cultivation, the calli were washed 5 times with medium containing 500 mg dm^{-3} carbenicillin. After washing, the calli were blotted dry with sterile *Kimwipes* and transferred to modified N6 medium containing 500 mg dm^{-3} carbenicillin and 50 mg dm^{-3} hygromycin for selection of transgenic calli. After double selection, each of 15 d, the well growing calli were transferred to Murashige and Skoog (1962; MS) regeneration medium containing 30 g dm^{-3} sucrose, 30 g dm^{-3} sorbitol, 2 g dm^{-3} casaminoacids, 2 mg dm^{-3} kinetin, 0.02 mg dm^{-3} naphthaleneacetic acid (NAA), 0.5 g dm^{-3} carbencillin, 50 mg dm^{-3} hygromycin and 4 g dm^{-3} *Gelrite*, while the pH was adjusted to 5.7. Again after double selection, each of 15 d, the regenerated calli were transferred to MS rooting medium containing 30 g dm^{-3} sucrose, 50 mg dm^{-3} hygromycin and 2 g dm^{-3} *Gelrite*, while the pH was adjusted to 5.7. After 10 d on MS rooting medium, the hygromycin-resistant plants were potted and grown to maturity at 30 °C and 16-h photoperiod with irradiance of 600 μmol m^{-2} s^{-1} in a greenhouse.

Bioassy analysis: For inoculation, compatible race of *Xanthomonas oryzae* pv. *oryzae* (Xo7435) was grown on media containing 1 % peptone, 1 % sucrose and 1.5 % agar at 30 °C for 2 d. A bacterial suspension was prepared by washing the cells from the culture slant with sterilized distilled water. Bacteria with a concentration of 10^8 colony forming units (cfu) cm^{-3} were used. The sense thaumatin-like protein transgenic rice was grown in plastic seedling cases (280 × 160 × 90 mm) in growth chamber for 3 weeks. The uppermost fully opened leaves of sense thaumatin-like protein transgenic rice were inoculated with bacterial suspension by double needles pricking method. In double needle pricking method, a wooden fork with one arm wrapped in the cloth and the other arm glued with a rubber piece containing a single needle was used for pricking in a way that rice leaf on each side of the midrib was singly pricked. Lesion lengths were measured at 3, 6 and 9 d after the bacterial inoculation.

Preparation of cytosolic and membrane protein fractions: A portion (170 mg) of rice leaf blade was suspended in 1 cm^3 homogenization buffer containing 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 1 mM dithiothreitol (DTT) and 1 mM phenylmethyl sulphonyl fluoride, and homogenized in a glass mortar. The homogenates were centrifuged at 3 000 *g* for 5 min, followed by the ultra centrifugation of the supernatant at 274 000 *g* for 15 min. Cytosolic fraction was obtained by collecting the supernatant. The pellet was washed with 0.4 cm^3 homogenization buffer followed by ultra-centrifugation at 274 000 *g* for 15 min. It was again resuspeded in 0.05 cm^3 membrane solubilizing buffer

containing 1 % Triton X-100, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA and 50 mM 2-mercaptoethanol, and incubated on ice for 30 min. After centrifugation at 274 000 *g* for 8 min, membrane fraction was obtained by collecting the supernatant (Komatsu and Hirano 1993).

Total protein concentration of cytosolic and membrane fractions was assayed using a standard assay kit (*Sigma-Aldrich*, St. Louis, MO, USA).

Western blot analysis: For Western blot analysis, sodium dodecylsulphate (SDS) sample buffer (Laemmli 1974) in 1:1 ratio was added to the cytosolic fraction (10 μg) before loading. The prepared samples were separated by SDS-PAGE and electroblotted onto a polyvinylidine difluoride (PVDF) membrane (*Pall*, Port Washington, NY, USA) using a semidry transfer blotter (Nippon Eido, Tokyo, Japan). The blots were blocked with 3 % gelatine in solution containing 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl. The anti-thaumatin like protein antibody (Konishi *et al.* 2001) was used as a primary antibody at a concentration of 1/1000 and incubated overnight. After washing, anti-rabbit IgG horse radish peroxidase (HRP, *Bio-Rad*, Hercules, CA, USA) antibody was used as a secondary antibody at a concentration of 1/3000 for 1 h. The PVDF membrane was stained by the HRP Colour Development Reagent (*Bio-Rad*).

Two-dimensional polyacrylamide gel electrophoresis: For two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), lysis buffer (O' Farrell 1975) with 1:1 ratio was added to cytosolic and membrane fractions (400 μg) before loading. The prepared samples were separated by 2D-PAGE (O'Farrell 1975), in the first dimension by isoelectric focusing tube gels and in the second dimension by SDS-PAGE. An isoelectric focussing tube gel of 11 cm length and 0.3 cm diameter was prepared which consisted of 8 M urea, 3.5 % acrylamide, 2 % Nonidet P-40, 2 % ampholines (pH 3.5 - 10.0 and pH 5.0 - 8.0), ammonium persulfate and tetramethylethylenediamine. Electrophoresis was carried out at 200 V for 30 min, followed by 400 V for 16 h and 600 V for 1 h. After the first dimension, SDS-PAGE was performed in the second dimension using 15 % polyacrylamide gels with 5 % stacking gels, followed by Coomassie brilliant blue (CBB) staining. The isoelectric point and molecular mass of each protein were determined using 2D-PAGE markers (*Bio-Rad*).

Image acquisition and data analysis: Stained gels from 3 independent experiments were analyzed with *PDQuest* software (*version 7.1*, *Bio-Rad*). Spot detection, spot measurement, background subtraction and spot matching were performed. Following automated spot detection, gel images were carefully edited. One gel image was selected as a reference followed by automated spot matching among the gels. The unmatched spots of the member gels were added to the reference gel. The amount of protein spot was expressed as the volume of that spot which was defined as the sum of the intensities of all the pixels that made up that spot. To correct the variability due to CBB staining, and to reflect the quantitative variations in intensity of protein spots, the spot volumes were normalized as the percentage of the total volume in all of the spots present in the gel.

N-terminal amino acid sequence analysis and homology search: Following separation by 2D-PAGE, proteins were electroblotted onto a PVDF membrane and detected by CBB staining. The protein spots were excised from the PVDF membrane and applied to the protein sequencer (*Procise 494*, *Applied Biosystems*). Edman degradation was performed according to the standard program supplied by *Applied Biosystems*. The amino acid sequences obtained were compared with those of known proteins in the databases:
Swiss-Prot (http://us.expasy.org/sprot),
PIR (http://pir.georgetown.edu),
GenPept (http://www.genelynx.org/cgi-bin/resource)
DDBJ (http://www.ddbj.nig.ac.jp/Welcome-e.html)
PBD (http://www.rcsb.org/pdb) using the Web-accessible search program in a *FastA* format.

Mass spectrometry analysis and database search: The protein spots were excised from gels, washed with 25 % methanol and 7 % acetic acid for 12 h and destained with 50 mM NH_4HCO_3 in 50 % methanol for 1 h at 40 °C. Proteins were reduced with 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 60 °C and incubated with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min. The gel pieces were minced and allowed to dry and then rehydrated in 100 mM NH_4HCO_3 with 1 pM trypsin (*Sigma-Aldrich*) at 37 °C overnight. The digested peptides were extracted from the gel pieces with 0.1 TFA in 5 % acetonitrile/water 3 times. The peptide solution, thus obtained was dried and reconcentrated with 0.03 cm^3 of 0.1 TFA in 5 % acetonitrile/water and then desalted with *NuTip C-18* pipette tips (*Glygen*, Columbia, MD, USA). The above peptide solution was mixed with α-cyano-4-hydroxynnamic acid. Matrix assisted laser desorption/ionization time of flight (*MALDI-TOF*) MS was performed using *Voyager PR* (*Applied Biosystems*, Framingham, MA, USA). The mass spectra were subjected to sequence databases search using Mascot software (*Matrix Science*, London, UK). For *MALDI-TOF* analysis, four criteria were used to assign a positive match with a known protein: *1*) the deviation between experimental and theoretical peptide masses should be less than 500 pg; *2*) at least four different predicted peptide masses needed to match the observed masses for an identification to be considered valid; *3*) the coverage of protein sequences by the matching peptides must reach a minimum of 10 %; *4*) the score that was obtained from the analysis with *Mascot* software indicates the probability of a true positive identification and must be at least 58 using rice full length cDNA database (*Version 20040709*) and at least 67 using *GenBank* database (*Version 20040722*).

Results and discussion

Leaf blades of 3-week-old rice seedlings of cultivar ZTS were inoculated with 10^8 cfu cm^{-3} of compatible strain Xo7435 of *X. oryzae* pv. *oryzae* and obvious symptoms were observed at day 9 after inoculation. Both lines 1 and 2 of antisense-thaumatin like protein transgenic rice were clearly diseased while line 1 of sense-thaumatin like protein transgenic rice was resistant as compared to the vector control plants. On the other hand, line 2 of sense thaumatin-like protein did not show much resistance (Fig. 1*A*). Lesion lengths of antisense-thaumatin like protein transgenic rice of both line were 11 cm each, while that of line 1 of sense thaumatin-like protein transgenic rice was 7 cm (Fig. 1*B*). To detect the overexpression of thaumatin-like protein, anti-thaumatin like protein antibody was used. Thaumatin-like protein was faintly expressed in antisense and vector control transgenic rice, while highly and moderately expressed in the lines 1 and 2 of sense transgenic rice, respectively (Fig. 1*C*).

It has been demonstrated that high level expression of PR proteins enhanced resistance to a variety of pathogens (Broglie *et al.* 1991). Thaumatin-like protein has proved antifungal activity, providing a base for the preparation of transgenic rice with resistance against various pathogens (Zhu *et al.* 1996). Overexpression of thaumatin-like protein gene in rice increased resistance to *R. solani*, the causal agent of sheath blight disease of rice (Datta *et al.* 1999). Based on these results and the present study, it is indicated that thaumatin-like protein plays an important role in resistance of rice against bacterial blight disease.

A total of 440 proteins were detected on 2D-PAGE,

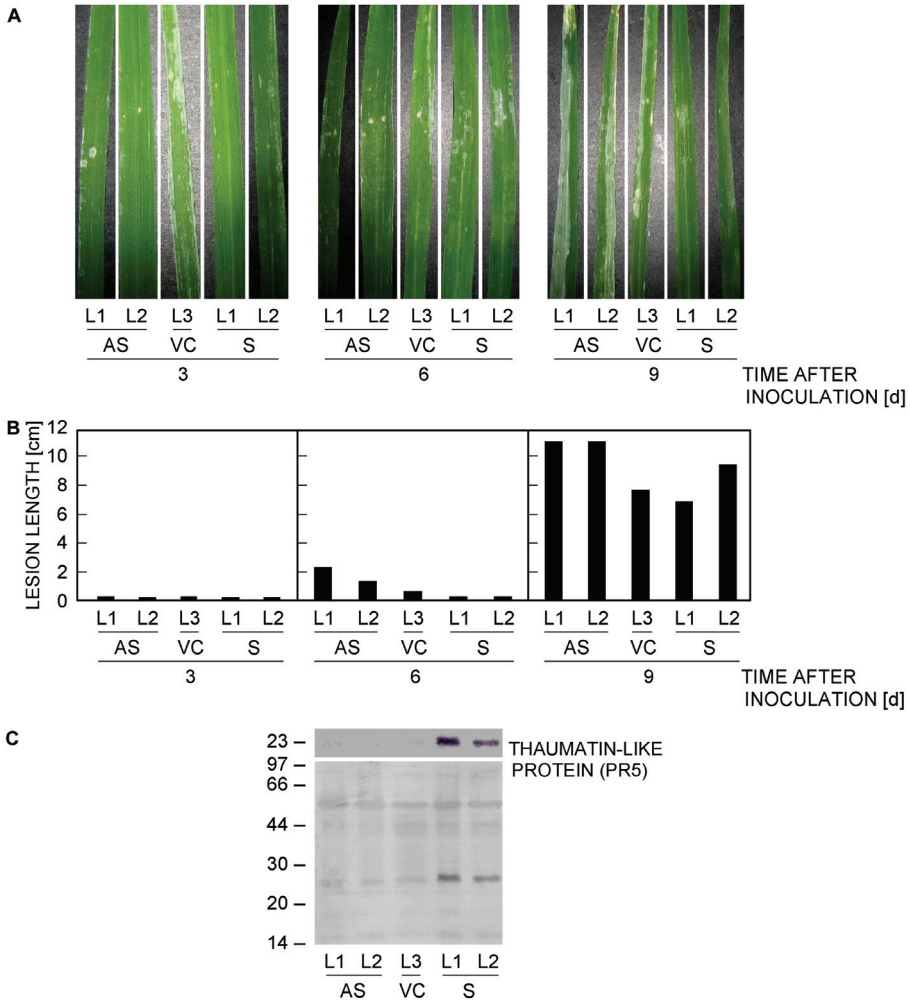

Fig. 1. Bioassays of sense and antisense thaumatin-like protein gene-transgenic rice. Leaf blades of 3-week-old rice seedlings were inoculated with compatible strain Xo7435. Photographs of the inoculated leaf blades of sense and antisense thaumatin-like protein transgenic rice were taken at 3, 6 and 9 d after Xo7435 inoculation (*A*) and the lesion lengths was determined (the mean lengths of lesion from 3 leaf blades; *B*). Cytosolic proteins from the leaf blades of antisense (AS), vector control (VC) and sense (S) thaumatin-like protein gene transgenic rice were fractionated, separated by SDS-PAGE and transferred onto PVDF membrane followed by Western blot analysis using anti-thaumatin like protein antibody (*C*).

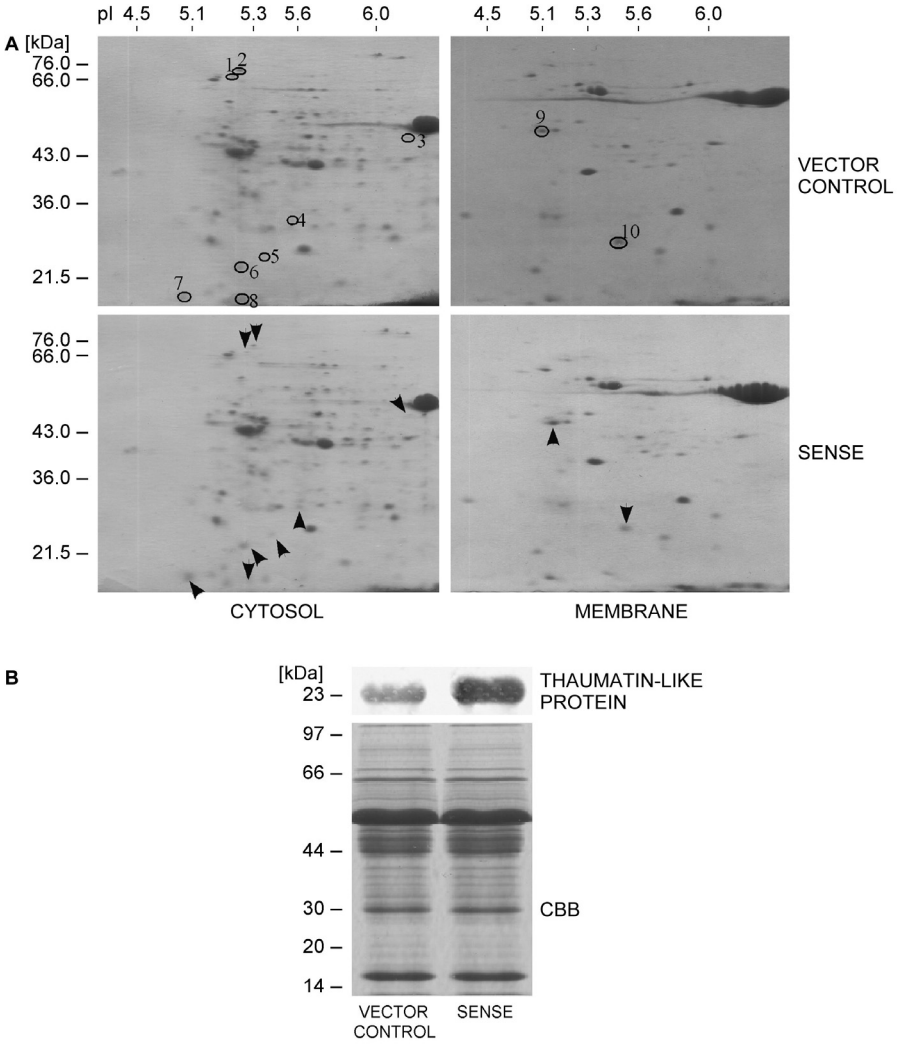

Fig. 2. Protein changes in sense thaumatin-like protein gene transgenic rice. Cytosolic and membrane proteins from the leaf blades of 2-week-old transgenic rice were fractionated, separated by 2D-PAGE and stained with CBB. *Up-ward arrows* indicate the positions of up-regulated proteins and *down-ward arrows* show the positions of down-regulated proteins, while *circles* represent the same proteins in vector control plants (*A*). Cytosolic proteins from the leaf blades were fractionated, separated by SDS-PAGE and transferred onto PVDF membrane followed by Western blot analysis using anti-thaumatin like protein antibody. CBB stained pattern represent loading control proteins (*B*).

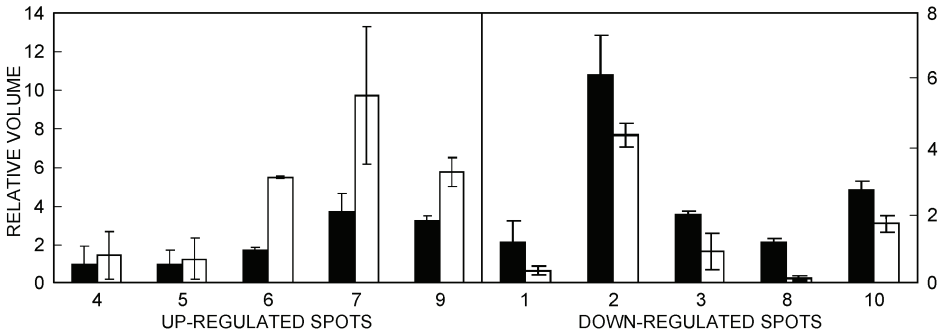

Fig. 3. Relative expression of changed proteins in sense thaumatin-like protein transgenic rice. Proteins from the leaf blades of 2-week-old rice seedlings were fractionated, separated by 2D-PAGE and stained with CBB. The changes in protein spots were calculated with *PDQuest* software and plotted as the relative volume of 10 spots. Means ± SE from 3 independent experiments. Black and white bars represent vector control and sense thaumatin-like protein transgenic rice, respectively.

where 10 proteins, including thaumatin-like protein, were differentially expressed. Thaumatin-like protein was up-regulated in the sense transgenic rice, and 4 proteins were up-regulated and 5 proteins were down-regulated as an effect of thaumatin-like protein overexpression (Fig. 2*A*). To detect the overexpression of thaumatin-like protein in particular, anti-thaumatin like protein antibody was used. In the sense thaumatin-like protein transgenic rice, this protein was 5 times up-regulated, whereas, mildly detected in vector control plants (Fig. 2*B*).

As a result of genetic manipulation, various changes occurred at protein levels, which have been successfully analyzed through proteomic approach. The induction of probenazole inducible protein 1 (PBZ1) was delayed in susceptible rice mutant dwarf 1(*d1*), when the proteins were analyzed using proteomic approach (Komatsu *et al.* 2004). Takahashi *et al.* (2005) reported that the proteomic analysis of cultured cells overexpressing *YK1*, a homologous gene of HC-toxin reductase, resulted in the up-regulation of several stress-related proteins such as osmotin-like protein and osr40c1. Thirty seven differentially expressed proteins were reported including proteins related to defence, metabolism, proteosome activities and stress in the rice resistant mutant, *cdr2* (Tsunezuka *et al.* 2005). These reports and the present study indicate that global changes at the protein level do occur in transgenic plants and mutants.

To analyze the protein expression using *PDQuest* software, proteins were grouped as either up-regulated or down-regulated spots. All of the 10 proteins exhibited differential expression in their relative volumes. Overexpression of thaumatin-like protein significantly up-regulated spots 6, 7 and 9, while spots 4 and 5 were moderately up-regulated. On the other hand, spots 1, 3 and 8 were significantly down-regulated, while spots 2 and 10 were moderately down-regulated (Fig. 3).

Proteomic analysis of transgenic rice overexpressed with *YK1* gene revealed that 5 proteins were up-regulated relative to control among 668 detected proteins by CBB (Takahashi *et al.* 2005). Tsunezuka *et al.* (2005) reported that 28 proteins were up-regulated and 9 proteins were down-regulated in the lesion mimic mutant *cdr2*. These results suggest that 2 proteins which are up-regulated and 3 proteins which are down-regulated may compliment each other as a down-stream effect of the overexpression of thaumatin-like protein, thus conferring resistance to the rice against bacterial blight disease.

Six proteins, 2-cys peroxiredoxin (spot 4), thaumatin-like protein (spot 6), glycine cleavage H protein (spot 7), salt induced protein (spot 8), ATP synthase B chain (spot 9) and oxygen evolving enhancer protein 2 (OEE2, spot 10) were identified (Table 1). Based on homology search, functions to the identified proteins were assigned. 2-Cys peroxiredoxin (spot 4) was up-regulated in this study. Peroxiredoxin reduces the oxidative damage to the chloroplast caused by oxidative stress (Dietz *et al.* 2002). It was reported that peroxiredoxin was up-regulated which played the role of balancing agent of oxidative stress to acquire resistance of rice against *X. oryzae* pv. *oryzae* (Mahmood *et al.* 2006). These results indicate that acquiring an optimum state of oxidative stress as per requirement of the plant is needed to get resistance in the thaumatin-like protein overexpressed transgenic rice.

Glycine cleavage H protein (spot 7) was up-regulated in the present study. Imin *et al.* (2006) reported that glycine cleavage H protein was up-regulated in the rice anthers, when treated with low temperature stress. Rir1a, a PR protein contained signal peptide cleavage site, was relatively rich in glycine and proline contents. Further, Rir 1a was similar in sequence to another pathogen induced protein Wir1 in wheat (Mauch *et al.* 1998). These results and the present study indicate that glycine cleavage H protein might be an important complimentary agent together with thaumatin-like protein to confer resistance to bacterial blight disease of rice.

Salt inducible protein (spot 8), which was predominantly induced by NaCl stress (Kim *et al.* 2004), was down-regulated in the present study. However, this protein was up-regulated in rice seedlings in response to drought stress (Ali and Komatsu 2006). These results suggest that resistance conferred by thaumatin-like protein overexpression against bacterial blight disease of rice is exploiting a pathway other than that exploited by abiotic stress, at least in terms of salt inducible protein.

ATP synthase B chain (spot 9) is the component of supercomplexes, which are parts of the photosystem 1 of the chloroplast and also play important roles in the mitochondria respiration (Eubel *et al.* 2003, Jilao *et al.* 2005). In the present study, ATP synthase was up-regulated, which might indicate that the respiration system of the rice was active and provided the energy required for resistance against bacterial blight of rice.

In the present study, oxygen evolving enhancer protein 2 (spot 10), an important component of photosystem 2, was down-regulated in thaumatin-like protein overexpressed transgenic rice. Photosystem 2 is also one of the sources of reactive oxygen species under biotic and abiotic stresses (Zou *et al.* 2005). Long term exposure of rice seedlings to abiotic stress would result into the decrease of oxygen evolving enhancer protein 2 as reported in the case of salt stress by Abbasi *et al.* (2004). Transgenic tobacco expressing a foreign calmodulin gene exhibited an enhanced production of active oxygen species (Harding *et al.* 1997). Based on these results and the present study, it is suggested the presence of oxidative stress in the thaumatin-like protein overexpressed transgenic rice.

Mahmood *et al.* (2006) noticed the up-regulation of thaumatin-like protein in the resistance interaction between rice and *X. oryzae* pv. *oryzae*. Further, the expression of ATP synthase B chain remained constant, and reduced in the susceptible interaction between rice and *X. oryzae* pv. *oryzae*. Rir1b, a PR protein possessed cleavage site in the hydrophobic N-terminal and a C-terminal part and was rich in glycine and proline, was important in the resistance of rice against rice blast fungus, *Pyricularia oryzae* (Mauch *et al.* 1998). Xo7435 is a compatible strain, which developed severe oxidative stress in the rice plant due to its pathological effect, resulting into

the down-regulation of oxygen evolving enhancer protein 2 (Mahmood *et al.* 2006). Further, 94 chloroplast associated genes, including oxygen evolving enhancer protein 2 transcripts, were down-regulated a resistance response of soybean plant against *Pseudomonas syringae* (Zou *et al.* 2005). In the present study, oxygen evolving enhancer protein 2 was down-regulated in both thaumatin-like protein overexpressed transgenic rice and after Xo7435 inoculation. These results suggest that oxidative stress plays a vital role in the attainment of resistance by thaumatin-like protein overexpressed transgenic rice against Xo7435.

A proteomic approach was performed to identify proteins from the rice leaf blade that were differentially expressed as a result of thaumatin-like protein gene overexpression. The effectiveness of PR proteins against fungal pathogens (Mauch *et al.* 1998) provided base for the production of resistant plants with genes for these proteins. Transgenic lines expressing thaumatin-like protein had moderate level of resistance against scab disease of fungus in wheat plant in greenhouse conditions (Anand *et al.* 2003). Further, Mahmood *et al.* (2006) noticed that the expression of thaumatin-like protein was higher in the resistant interaction between rice and

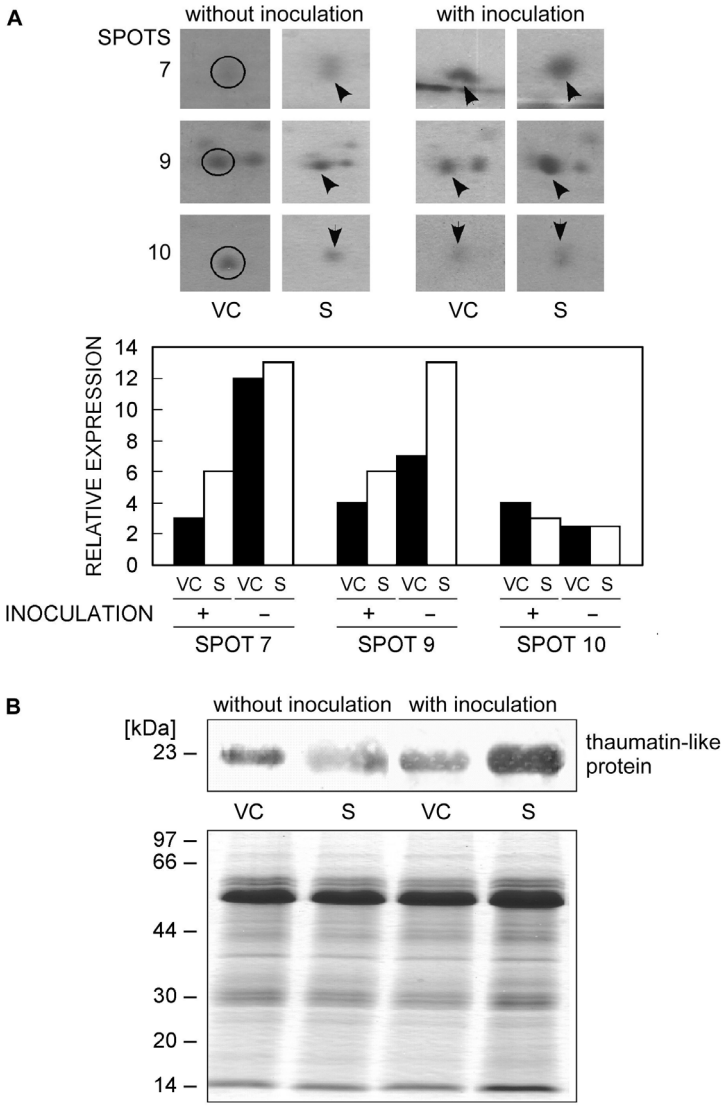

Fig. 4. The effect of Xo7435 inoculation on the proteins modulated by thaumatin-like protein overexpression. Leaf blades of 2-week-old vector control and sense thaumatin-like protein transgenic rice seedlings were inoculated with compatible strain Xo7435 of *X. oryzae* pv. *oryzae* at a concentration of 10^8 cfu cm^{-3}. 3 d after inoculation, cytosolic and membrane proteins were fractionated, separated by 2D-PAGE and stained with CBB. *Upward arrows* indicate the positions of up-regulated proteins and *downward arrows* show the positions of down-regulated proteins in the sense thaumatin-like protein transgenic rice, while *circles* represent the same proteins in vector control plants. The changes in protein expression of the spots changed by Xo7435 inoculation were calculated with *PDQuest* software and plotted as the relative intensities of these spots (*A*). The expression of spot 6 by Western blot analysis and its relative abundance changed by Xo7435 inoculation (*B*). CBB stained pattern represent loading control proteins.

Table 1. The changed proteins in rice leaf blade in the sense thaumatin-like protein transgenic rice. N-terminal amino acid sequences were determined by Edman sequencing; pI and Mr values were derived from 2D-PAGE spot positions (exp/the - experimentally determined/theoretical values; NSM - not significantly matched, ND - not detected).

Spot No.	pI (exp/the)	Mr (exp/the)	Sequence	Homologous protein [%]	Accession No.
4	5.6/5.4	30/26	N-AGGVDDAPLV	2-Cys peroxiredoxin (100)	CAJ01693
6	5.4/4.7	21/17	N-ATFTITNRXS	thaumatin-like protein (90)	P31110
7	4.9/4.7	18/17	N-STVLDGLKYS	glycine cleavage H protein (90)	P93255
8	5.2/6.0	16/52	N-TLVKIGPWGG	salt induced protein (100)	AF001395
9	5.1/4.6	40/19	N-KELDEEKQ	ATP synthase B chain (100)	Q9K6H1
10	5.5/ND	23/22	N-AYGEAANVF	oxygen evolving enhancer protein 2 (100)	P80661
1	5.2	66	N-blocked (MS)	NSM	-
2	5.2	70	N-blocked (MS)	transketolase	AK067452
3	7.0	55	N-blocked (MS)	glycerol aldehyde dehydrogenase	S33872
5	5.4	23	N-N-blocked (MS)	NSM	-

X. oryzae pv. *oryzae*. PR proteins have been found to impart resistance to rice against sheath blight disease (Datta *et al.* 1999). In the present study, a moderate level of resistance was observed in the sense thaumatin-like protein transgenic rice. Based on these results, the effectiveness of thaumatin-like protein overexpression to confer a moderate level of resistance in rice against bacterial blight disease is suggested. It is further suggest that in both thaumatin-like protein overexpressed transgenic rice and its resistant interaction with *X. oryzae* pv. *oryzae*, the defence, oxidative stress and energy metabolism mechanisms are in active states of functions that support each other to give resistance to rice against bacterial blight disease.

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