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Separation of proteins from stressed rice (*Oryza sativa* L.) leaf tissues by two-dimensional polyacrylamide gel electrophoresis: Induction of pathogenesis-related and cellular protectant proteins by jasmonic acid, UV irradiation and copper chloride

We have used three kinds of stresses, including the signaling compound jasmonic acid, an environmental stressor, UV irradiation, and a heavy metal salt copper chloride, to study changes in the protein patterns in rice (*Oryza sativa* L.) leaf tissues using two-dimensional polyacrylamide gel electrophoresis. However, instead of using lysis buffer containing urea (O'Farrell, *J. Biol. Chem.* 1975, 250, 4007–4021) for extraction of proteins from rice seedling tissues, we used Tris-HCl buffer (commonly used for extraction of proteins for separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) for extraction of proteins and resolved these extracted proteins by the usual method of O'Farrell. Furthermore, the induction of a large number of proteins was clearly observed over controls. No spots corresponding to these induced proteins were found in the control experiment, indicating qualitative changes in protein patterns after various stress treatments. A total of 12 out of 13 proteins could be *N*-terminally sequenced from jasmonic acid-treated rice leaf tissues, and one protein was sequenced from UV-irradiated leaf tissues. These proteins showed high homology to pathogenesis-related (thaumatin-like protein, a PR5 class protein; a beta-1,3-glucanase precursor; an intracellular PR protein encoded by PBZ1 gene, and an antifungal protein) and cellular protectant (glutathione transferase, EC 2.5.1.18; and ascorbate peroxidase) proteins, from plants, including rice. Results presented here suggest a role for jasmonic acid in the self-defense mechanisms of rice plants.

Keywords: Two-dimensional polyacrylamide gel electrophoresis / Rice / Jasmonic acid / UV irradiation / Copper chloride / Tris-HCl
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1 Introduction

It is common practice to resolve and separate proteins, extracted using lysis buffer from plants and animal tissues, by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) following the well-established procedure of O'Farrell from 1975 [1]. Highly reproducible and clean separation of proteins is still somewhat of a problem with traditional 2-D PAGE using lysis buffer for extraction, especially in plants. In the case of rice plants, containing fibrous matter and a large amount of phenolics and pigments, especially after the plants are stressed, this leads to not very highly reproducible gel patterns using 2-D PAGE, which is a prerequisite for experiments involving detailed and precise protein induction and suppression.

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Abbreviations: JA, jasmonic acid; TLP, thaumatin-like protein

Secondly, we wanted to find a method of separating proteins by 2-D PAGE after extraction using commonly used homogenization buffers, like Tris-HCl. Combining the Tris-HCl homogenization buffer for extraction of soluble proteins from rice plant leaf tissues and separation in the first dimension of O'Farrell [1], and second dimension by standard SDS-PAGE, we present results demonstrating beautifully separated proteins from rice leaf tissues, which correspond clearly to the induced polypeptide bands on SDS-PAGE.

Rice, being an important staple crop in most of southeastern Asia, both economically and socially, was used for these present experiments. No detailed studies are available to date on protein patterns including induction and/or suppression in rice after stress or phytohormone treatments. Recently, the role of jasmonic acid (JA) as a potent inducer of rice phytoalexin production [2] and in induction of a methyltransferase, naringenin 7-*O*-methyltransferase (catalyzes the last step in biosynthesis of rice phytoalexin sakuranetin) in rice leaves was reported [3]. The abiotic stressors, UV irradiation, and a heavy metal

salt copper chloride (CuCl_2) also induced the phytoalexin sakuranetin in rice [2]. As the rice plant is able to respond to a wide variety of stresses, biotic and abiotic, with changes in the morphology and biochemistry, we planned and conducted experiments in rice using these stressors, with the specific aim to identify changes in protein patterns in rice leaves. The method used was a slight modification of the O'Farrell 2-D PAGE technique, using Tris-HCl buffer for homogenization, loading, and separation on the first-dimensional IEF gels. Applying this modification to the separation of rice leaf proteins, we present results on the induction of thirteen clearly identifiable proteins in JA-treated rice leaves, some of which are also induced after UV or CuCl_2 treatments. Amino acid sequencing and homology studies on these induced proteins revealed their possible roles in stressed rice plants, such as cellular protection or pathogenesis-related.

2 Materials and methods

2.1 Plant material

Rice (*Oryza sativa* L. cv. Hitomebore) were cultivated in the greenhouse. At the sixth leaf stage, the fourth and fifth leaves were detached for use as experimental material. Detached rice leaves were treated with 500 μM JA, 500 μM CuCl_2 , and short wave UV irradiation as described previously [2, 3], except that in the present experiments leaf segments were floated on test solutions in Petri dishes. Briefly, rice leaves were cut into 2 cm segments and floated on 5 mL of the test solution or control, in a 6 cm clean glass Petri dish, and incubated for 72 h under continuous illumination (fluorescent light) in a growth chamber at 26°C. For UV irradiation (short wave), rice leaf segments were irradiated for 20 min using a single Hitachi germicidal lamp (15 W) at a distance of 20 cm, and incubated as above. Immediately after incubation, leaf segments were blot-dried on a Kimwipe tissue and stored at -80°C until analyzed for induction of proteins (or changes therein).

2.2 Chemicals

JA was prepared by deesterification of ($\pm$)-methyl jasmonate (Harmann and Reimer, Holzminden, Germany) as described previously [2]. JA and CuCl_2 were applied as solutions in distilled water. The protein dye reagent, TEMED, ammonium persulfate, acrylamide, *N,N'*-methylenebisacrylamide, and urea were from Bio-Rad (Richmond, CA, USA). Ampholine, pH 3.5–10.0 for IEF, and LMW electrophoresis calibration kit were from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of reagent grade.

2.3 Preparation of crude protein

For preparation of crude protein extracts of rice leaves, approximately 300 mg of the treated rice leaves were homogenized in 1.2 mL of 0.2 M Tris-HCl buffer, pH 7.8, as described [3]. The homogenates were centrifuged at $18\,500 \times g$ for 5 min, with the resulting supernatant filtered through a 50 μm nylon mesh and used as crude protein preparation for SDS-PAGE or 2-D PAGE analysis. The crude protein extract was used immediately to determine the protein concentration, with the Bio-Rad protein assay dye reagent and BSA as standard, and by the method of Bradford [4], and frozen at -20°C for 2-D PAGE analysis.

2.4 Gel electrophoresis

The concentration of crude protein extracts was first determined by the Bradford method [4], and then equal amounts (13.5 μg) were electrophoresed on 13.5% SDS-PAGE [5]. Briefly, after centrifugation, 78 μL supernatant was mixed with 22 μL blue juice (9% v/v 2-mercaptoethanol, 22.7% w/v sucrose, 4.5% w/v SDS and 0.01% w/v bromophenol blue), and samples were heated in a boiling water bath for 30 s. Separated polypeptides on the gel were visualized by staining with Coomassie Brilliant Blue (CBB). For 2-D PAGE, isoelectric focusing (IEF) in the first dimension was combined with SDS-gel electrophoresis for the second dimension [1] with some modifications. IEF was carried out in a glass capillary tube of 13 cm length and 3 mm ID. Briefly: the gel solution consisted of 10% NP-40, 30% w/v acrylamide, 9.5 M urea, 10% ammonium persulfate, and 2% carrier ampholytes (pH 3.5–10). Equal amounts (35 μg) of crude protein extract were loaded directly onto the IEF-capillary gel (cathodic side) without dissolving in the sample buffer. The sample overlay buffer consisted of 20 μL of 1/10 lysis buffer. Electrophoresis was carried out at 200 V for 30 min, followed by 400 V for 16 h and 800 V for 1 h. SDS-PAGE in the second dimension was performed with 13.5% separation gels and 5% stacking gels at a constant current of 35 mA. A 15×15 cm 2-D PAGE gel was used for separation in the second dimension. Separated proteins were stained with CBB, destained, and dried using a vacuum gel dryer on Whatman No. 3 filter paper.

2.5 N-terminal amino acid sequencing analysis

Following separation by 2-D PAGE, the proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Fluorotrans; Pall, Tokyo, Japan), using a semidry blotter (ATTO, Tokyo, Japan). The stained protein spots were excised from the PVDF membrane and

placed directly into the cartridge for sequencing on an Applied Biosystems (Foster City, CA, USA) 494 protein sequencer. Edman degradation was performed according to the standard program supplied by Applied Biosystems.

3 Results and discussion

3.1 Stress-induced changes in rice leaf protein patterns visualized by SDS-PAGE and 2-D PAGE

In preliminary experiments, SDS-PAGE analysis was carried out using crude protein extracts (13.5 μ g protein in each lane) from rice leaves treated with 500 μ M JA, 500 μ M CuCl_2 and short-wave UV irradiation to identify changes in protein patterns (Fig. 1). In previous studies [2, 3, 6] it was found that at concentrations of 500 μ M (although JA was also effective at lower concentrations of

100 μ M), JA is able to strongly elicit the phytoalexin response and thus was used at this concentration in this study. As can be seen from Fig. 1, JA, CuCl_2 , and UV irradiation cause reductions in the amounts of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit, (LSU, *ca.* M_r 49 000) and small subunit, (SSU, *ca.* M_r 15 000), as well as induction of numerous polypeptides marked by dots on the right-hand side of lane 2 (JA-treated), as compared with controls. RuBisCO (both LSU and SSU) decreases in stressed rice leaf tissues (seen in Fig. 1), consistent with results obtained in various plant genera treated with JA [7]. Thus JA, including the other two stress agents, CuCl_2 and UV, cause damage to RuBisCO, which is most probably due to a direct or indirect generation of reactive oxygen intermediates [8], or possibly due to action of these stresses, through secondary signaling pathways, at the level of gene expression.

However, as the polypeptide bands that were induced after various stress treatments were difficult to distinguish as single bands, due to the high intensity of proteins above or below them, it was necessary to separate these proteins using high resolution 2-D PAGE. Here, at the step where the crude protein extract is usually obtained (or homogenized) in lysis buffer [1], or if the sample is in SDS (which is normally incompatible with IEF), diluted into a solution containing at least an 8-fold excess of non-ionic or zwitterionic detergent prior to IEF, we electrophoresed the crude protein extract as such (in Tris-HCl buffer, pH 7.8) on the IEF gel [1]. After IEF and SDS-PAGE on a 13.5% polyacrylamide gel (13.5% SDS-PAGE could resolve the proteins clearly; Fig. 1), we were surprised to obtain a clean resolution of proteins (Fig. 2 A–D). Moreover, this separation and resolution of proteins was highly reproducible, and was subsequently used for obtaining a large amount of protein for *N*-terminal amino acid sequencing. For each sample, including control, three independent runs were carried out to confirm the presence of the induced protein spots. Then, only 6–8 gels per treatment were run, and separated proteins were transferred to PVDF membranes for *N*-terminal amino acid sequencing.

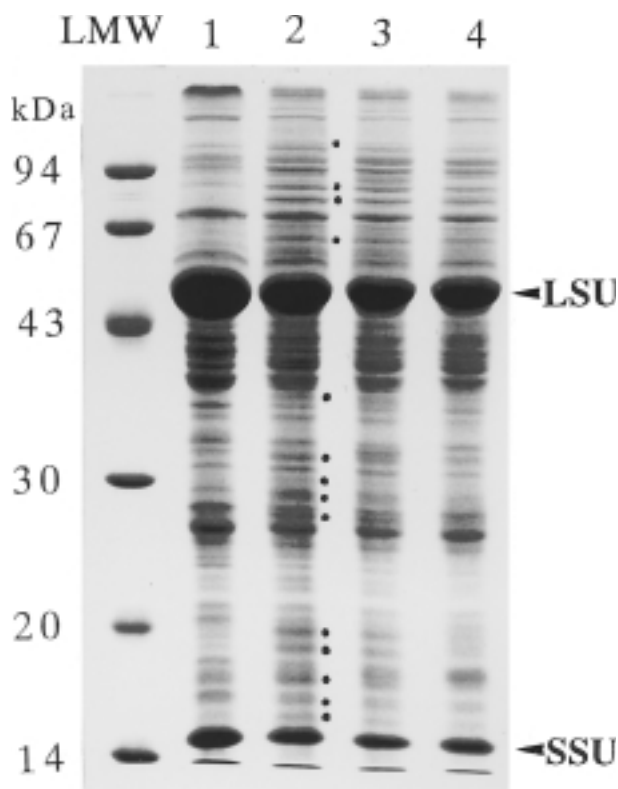


Figure 1. Changes in polypeptide patterns in rice leaves (lane 1, untreated control) treated with (2) 500 μ M JA, (3) 500 μ M CuCl_2 , and (4) short wave UV-irradiation, as visualized on 13.5% polyacrylamide gels. Approximately, 13.5 μ g protein was loaded onto each lane. Black dots on the right hand side of lane 3 indicate the induced polypeptide bands over untreated controls. Lanes (3) and (4) also show induction of some of these polypeptide bands. Low molecular weight markers (LMW) are indicated on the left hand side in kDa. The gels were stained with Coomassie brilliant blue.

From the 2-D PAGE patterns in Fig. 2A–D, we could clearly separate and identify the induced proteins, ranging from low to high molecular mass (14–94 kDa). Proteins extracted from untreated rice leaves from the same time period as used for treatment, were used as control for comparative purposes (Fig. 2A). The image analysis in this study was performed visually, and the changes observed are qualitative in nature. Note that only those proteins that were visually identifiable as not present in the control, or present at very low levels (protein spot JA 10 and 11, Fig. 2B), were selected in this present study; how-

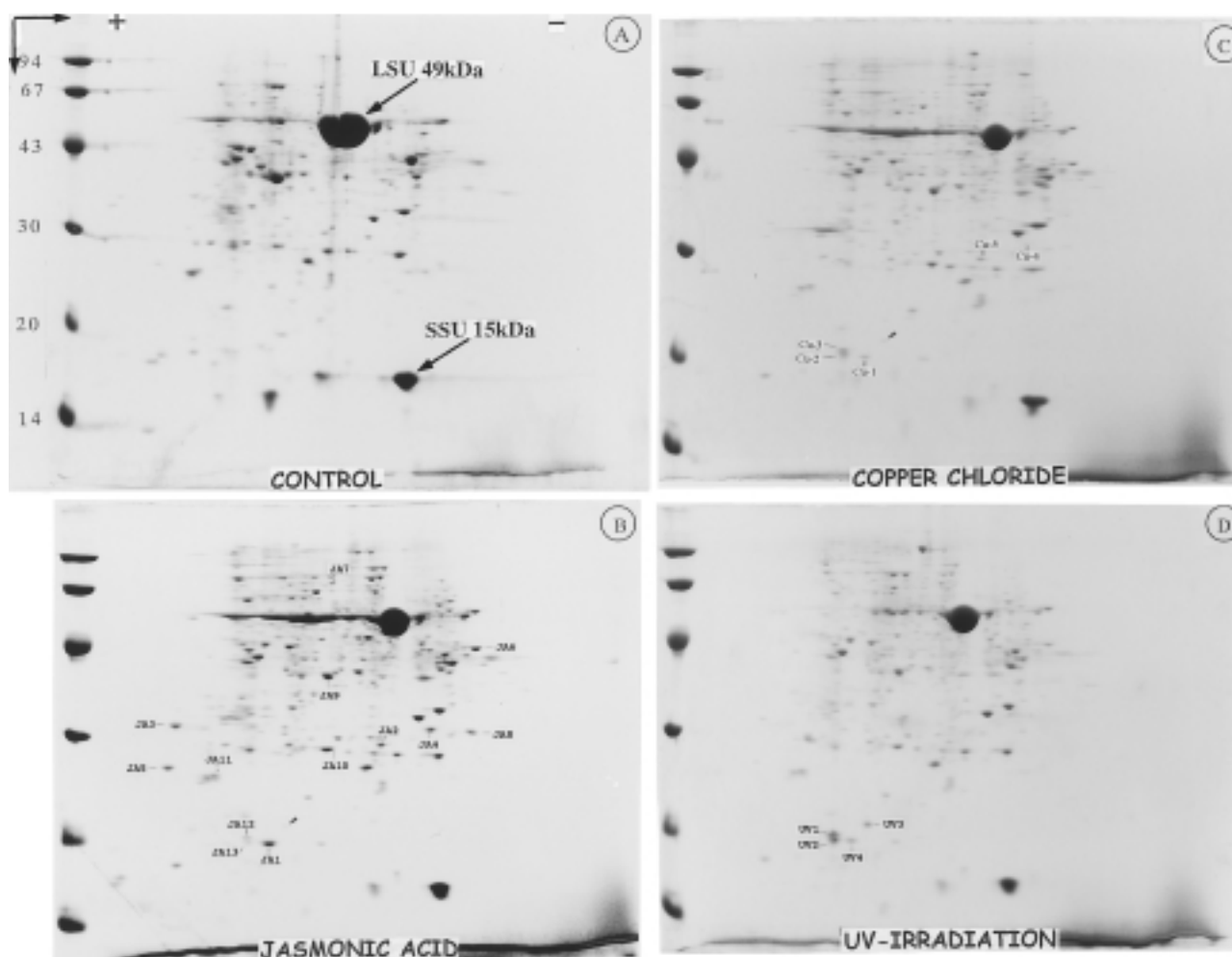


Figure 2. Coomassie stained 2-D PAGE gels of separated rice leaf proteins. (A) Untreated control, (B) treated with JA, (C) treated with CuCl_2 , and (D) after UV-irradiation. The separated proteins were obtained from the first-dimensional IEF gels with a pH range of 3.5 to 10, as described in Section 2. Approximately, 35 μg protein in Tris-HCl buffer were directly loaded onto the IEF gels for each treatment and control, and electrophoresed according to [1]. Numbered arrows JA 1–13 in (B), Cu 1–5 in (C) and UV 1–4 in (D) indicate induced proteins over control. The arrowheads in (B) and (C) refer to corresponding positions of the UV 3 protein. RuBisCO LSU and SSU are marked as 49 kDa and 15 kDa in (A), respectively. First dimension (left to right): IEF; (top to bottom): SDS-PAGE. Molecular mass markers in kDa are indicated on the left hand side in (A).

ever, some high molecular weight proteins induced after various treatments were not studied further in this experiment. Also, only these prominent proteins were further taken for *N*-terminal amino acid sequence analysis. The maximum number of proteins that were induced over controls were observed in JA-treated rice leaf tissues (Fig. 2B), although some proteins were common in CuCl_2 and UV-stressed leaf tissues (Fig. 2C, D). These proteins are numbered JA 1–13 (for JA-treated leaf segments), Cu 1–5 (for CuCl_2 -treated leaf segments), and UV 1–4 (for UV irradiated leaf segments) in Fig. 2 A–D. As the control gel (Fig. 2A) is slightly compressed in the x-axis region (from pH 5.0 to 7.0), three independent control gels were run to confirm that the induction of proteins is after stress treat-

ment only (data not shown). That these proteins are not the degradation of the LSU of RuBisCO, which first comes to mind observing drastic reductions in RuBisCO LSU, was confirmed by anti-RuBisCO LSU antibody experiments (data not shown), and also by the fact that amino acid sequencing of these induced proteins showed no homology to LSU sequences. Then, what are these induced proteins, and why are they being induced after stress treatments, and in particular after the phytohormone and signaling molecule JA in rice [9]? Is it a general response in rice against stress (some of the induced proteins being common with other stressors) or, as seen from the number and kinetics of induction in JA-treated tissues, a specific response to JA? Because JA and its

amino acid conjugates have been shown to be present in rice tissues, and endogenous levels are increased after stress [10], it is more likely that JA has a specific role in rice plant, probably as a stress modulating compound. However, the precise role(s) for JA in the rice plant remain to be established, and are currently under investigation in our laboratory.

3.2 *N*-terminal amino acid sequence analysis of induced proteins

Further investigations into the nature of these induced proteins was carried out by *N*-terminal amino acid sequencing. As the largest number of proteins (as well as relative amounts) induced were from JA-treated rice leaf tissues, and as some of these proteins were common after CuCl₂- and UV-treatment, sequence analysis was performed on all the JA-induced proteins (numbered JA 1–13). However, one low molecular weight protein, which was prominent in the UV-irradiated leaf tissues (UV 3), but only faint in CuCl₂-treated leaf tissues, and almost negligible to absent in JA-treated leaves, was taken for *N*-terminal sequencing (Fig. 2). The *N*-terminal amino acid

sequencing results are presented in Table 1. Out of a total of 14 proteins sequenced, one was blocked with no amino acid residue detected (JA 9), two were possibly blocked (JA 4, 7), seven were previously reported from rice (JA 1, 2, 3, 10, 11, 12, 13, and UV 3), one was a new protein from rice (JA 5), and two were unidentified, having a low sequence homology (JA 6, 8). Among these *N*-terminal sequenced proteins, four proteins are pathogenesis-related as revealed by sequence homology: (JA 1 (thaumatin-like protein), JA 3 (*Oryza sativa* beta-1,3-glucanase precursor; OSU72251_1(U72251|pid:g4097940), JA 5 (antifungal protein/s-fragment), and JA 12 and UV 3 (rice mRNA for probenazole inducible protein PBZ1; D38170_1(D38170|pid:g1215812). Thaumatin-like proteins are known as PR-5 proteins [11]. To date, more than a dozen members of the superfamily of thaumatin-like proteins are known, but their enzymatic function remains unknown, although a direct defensive function has been demonstrated for some proteins in this superfamily [11].

This is also the first report on induction of thaumatin-like protein (TLP) by JA in rice, as also by CuCl₂-treatment (Cu 1) and after UV irradiation (UV 4), though at consider-

Table 1. *N*-terminal amino acid sequences and percentage homology of identified proteins from JA-treated and UV-irradiated rice leaves

Protein	Sequence	Molecular mass (kDa)	% Homology
JA- 1	ATFTITNRASFTVRPAATPVGGGVW (Thaumatococcus-like protein)	19	91.7
JA- 2	SPMKLYGSTL (Glutathione transferase)	28	70.0
JA- 3	IGVAYGVIGNNLPRPSDVVQLYKQN (Beta-1,3-glucanase precursor)	31	88.0
JA- 4	SGARPRPIQDRIY	26	–
JA- 5	ATFTITNKAQITVCAAAPVFGGG (Antifungal protein/s fragment)	25	73.9
JA- 6	AGAEQSEALKSTALQD (Carbon catabolite derepressing protein kinase)	40	63.6
JA- 7	SPGLASVAG	69	–
JA- 8	APPRPPKPLDDSCDDQMSKQ (Unidentified)	30	–
JA- 9	n.d.	36	–
JA-10	AKNYPVQSAEYREALEQQK (Ascorbate peroxidase)	27	81.2
JA-11	AGGVDDAPLVGNKAPDFDAEQQFQ (Parvalbumin)	24	87.0
JA-12	APAWVSDEHAVVQAERLWK (Probenazole-inducible protein PBZ1)	20	90.0
JA-13	APVSISDERAVSVQAERVVK (Ubiquinol-cytochrome <i>c</i> reductase)	19	93.3
UV-3	APAFVSDERAVAVSVERLKKVCLDV (Probenazole-inducible protein PBZ1)	21	90.0

n.d., no amino acid residues detected

ably lower levels. The induction of a TLP by JA in rice comes as no surprise, if we agree to a role for JA as a stress-modulating compound, including its involvement in the hypersensitive response in rice. The role for this protein in rice will only be established after further purification and functional characterization. When this paper was under review, a just published report by Datta *et al.* [12] indicated that overexpression of TLP resulted in enhanced resistance to *Rhizoctonia solani* in rice compared to control plants. This answers the question about the role of TLP in rice. However, we are further interested in how JA causes induction of this protein, and whether JA is involved in the signal transduction pathway leading from pathogen recognition and expression of TLP gene and its deployment against pathogen attack.

Another interesting observation is on the induction of two different proteins in rice, the JA 12 and the UV 3. These two proteins, though only slightly different in their *N*-terminal amino acid sequences, show high homology to the PBZ1 gene product [13] from rice (Table 1). Furthermore, whereas JA induces the JA 12 protein prominently, UV irradiation specifically induces the UV 3 protein (Fig. 2). On the contrary, UV 1 is the same as JA 12, but is more strongly induced by UV in rice (Fig. 2). Thus, in rice at least two PBZ1 gene products are present as seen from the 2-D PAGE results (Fig. 2B–D). The PBZ1 gene (encoding an intracellular PR protein) which was recently identified as a probenazole-inducible gene in rice [13] was suggested to be involved in a defense response of rice [14]. Its induction by JA suggests a role of JA in the defense response of rice, which is our idea in this paper. However, the stronger induction of PBZ1 by UV irradiation and also after CuCl_2 treatment suggests a role of this PBZ1 gene product against not only biotic, but also abiotic stress. As to why we can observe two PBZ1 gene products in rice specifically after abiotic stresses remains unclear at present. As also argued recently, the precise role of this gene in defense in rice remains to be established [14].

On the other hand, it was observed that JA strongly induces the accumulation of glutathione transferase (EC 2.5.1.18; JA 2) and ascorbate peroxidase (JA 10), which are known to be involved in cellular protection mechanisms [15]. Members of the glutathione transferase family are known to be strongly induced by H_2O_2 from the oxidative burst, among varied functions, detoxifying lipid hydroperoxides generated by the reactive oxygen intermediates [16]. The induction of glutathione transferase by JA in rice leaves is also a first report. It is suggested that these cellular protectant proteins, including glutathione transferase and ascorbate peroxidase, are induced in JA-treated rice leaves (also by CuCl_2 -treatment, Cu 5) to

counteract deleterious effects of the reactive oxygen intermediates generated in the rice cells. JA as well as CuCl_2 and UV, are further speculated to cause an increase in the reactive oxygen intermediates in rice cells, either through breakdown of the photosynthetic apparatus, or through lipid peroxidation after these treatments. This is speculative at best; however, induction of these two cellular protectant proteins in response to JA suggest such a role in rice plant.

4 Concluding remarks

Considering the fact that jasmonates are putative plant growth substances and also proposed to be stress-related compounds [7], what is the physiological significance of our observations? One suggestion is that JA and its amino acid conjugates [2, 6, 8, 10] are not only involved in mediating rice phytoalexin production, but are also part of a general stress response in rice plants, either to biotic or abiotic stress, including disease, by inducing pathogenesis-related and cellular protectant proteins. The strong induction of pathogenesis-related proteins can be attributed to the fact that JA is a potential signal molecule mediating defense responses in plants. On the contrary, however, the observation on accumulation of cellular protectant proteins by JA in rice leaves indicates that reactive oxygen intermediates are generated in response to exogenous JA treatment and play a role in rice plant self-defense mechanisms. We are presently undertaking a study to obtain cDNA clones for the gene encoding some of these induced proteins in rice and to study their expression and precise function in response to exogenous JA and after various abiotic stresses. This study also provides the first report on induction of TLP and the PBZ1 gene product, an intracellular PR protein by the phytohormone and signaling molecule JA in rice. Recently, the dual role of jasmonates in plant defense and development has been reviewed; over the past decade there has been increasing interest in this compound (JA, including MeJA) and its role in plants [17]. Information on the variety and nature of proteins induced in the rice plant (after JA, as also after abiotic stresses) will further our knowledge on the role JA plays during normal and/or abnormal physiological processes during rice plant growth and development. And, as argued in the final sentences of the review of Creelman and Mullet [17], the deployment of JA-inducible genes as part of the complex plant defense system will be a topic of intense future study, which is also true for rice and has just been shown using TLP overexpression [12]. Finally, this study provides a new way of looking at jasmonate-effected response in rice plants, and sets out to gain a better understanding of the role of this multifunctional compound JA in rice.

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