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Light/Dark Responsiveness of Kinetin-Inducible Secondary Metabolites and Stress Proteins in Rice Leaf

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Abstract

Kinetin (KN) is an inducer of rice (*Oryza sativa* L.) defense/stress responses, as evidenced by the induction of inducible secondary metabolite and defense/stress protein markers in leaf. We show a novel light-dependent effect of KN-triggered defense stress responses in rice leaf. Leaf segments treated with KN (100 μM) show hypersensitive-like necrotic lesion formation only under continuous light illumination. Potent accumulation of two phytoalexins, sakuranetin, and momilactone A (MoA) by KN that peaks at 48 h after treatment under continuous light is completely suppressed by incubation under continuous dark. Using two-dimensional gel electrophoresis we identified KN-induced changes in ribulose-1, 5-bisphosphate carboxylase/oxygenase, energy- and pathogenesis-related proteins (OsPR class 5 and 10 members) by N-terminal amino acid sequencing and mass spectrometry. These changes were light-inducible and could not be observed in the dark (and control). The present results provide a new dimension (light modulation/regulation) to our finding that KN has a potential role in the rice plant self-defense mechanism.

Key words: cytokinin, defense/stress related proteins, mass spectrometry, phytoalexins, proteomics

Introduction

The plant hormones cytokinins are a group of plant redifferentiation-inducing hormones involved in regulating a myriad of plant growth and development processes, including senescence. Our study involves investigating the rice (*Oryza sativa* L.) plant self-defense mechanisms (for review see Jwa et al. 2006). Rice has been termed as "a cornerstone for cereal food crop proteomes" (Agrawal and Rakwal 2006; Agrawal et al. 2006) and the "Rosetta stone for crop genomics" by Robin Buell, lead investigator for TIGR (The Institute for Genomic Research). Novel information on KN-mediated stress responses in rice was reported previously in 2003, working on the possible role of a cytokinin, kinetin (KN) in rice self-defense mechanism (Rakwal et al. 2003). Combined with our initial study and the multifaceted aspect of cytokinin action and function in both plants and animal systems, we had preliminary evidence on cytokinins, at least KN, showing that KN is a novel activator of rice plant defense/stress response pathway (Rakwal et al. 2003).

In the previous study, Rakwal and co-workers (Rakwal et al. 2003) noticed that the effect of KN in rice seedling leaves was different under light and dark incubation. To further investigate how light modulates KN-triggered responses in the leaves, the present study was carried out by documenting the direct effects of KN on leaf morphology after incubation under continuous light or darkness. Two experiments were carried out at the molecular level. In the first step, we looked at the levels of marker secondary metabolites in the leaves. In the second step, we examined in detail the changes in protein profiles after KN treatment by two-dimensional gel electrophoresis (2-DGE). Our results provide new evidence for light-regulated KN response in rice.

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Materials and Methods

Plant material and treatment

Rice (*Oryza sativa* L. japonica-type cv. Nipponbare) seedlings were grown for two weeks under white fluorescent light (wavelength 390-500 nm, 150 $\mu M/m^2/s^1$/, 12 h photoperiod) at 25 ℃ and 70% relative humidity as previously reported (Rakwal et al. 2003; Cho et al. 2006). Leaf segments (about 2 cm long) were cut with clean scissors and placed on KN [Sigma, 100 μM concentrated solution in Milli Q (MQ, Millipore, Waters)] solution in sterile plastic Petri dishes, which were immediately transferred to a growth cabinet (at 25 ℃, with continuous light of 150 $\mu M/m^2/s^1$/). For the dark treatment, lights were switched off in the same growth chamber. Leaf segments floated on MQ water served as an appropriate control in these experiments. The sampled leaf segments were frozen at -80 ℃ until further analyses. Two independent experiments were carried out and leaves were pooled from the two bioreplicates.

Phytoalexins determination

Leaves (50 mg) were extracted with 80% methanol (total volume of 5 ml) by boiling for 5 min. Three microliters of the crude extract were injected onto a high-performance liquid chromatography (HPLC) separation system and analyzed by a LC-tandem mass spectrometry (LC-MS/MS) technique exactly as previously described (Jung et al. 2005).

Preparation of total protein extract and 2-DGE

For extraction of total soluble protein for 2-DGE, leaves (ca. 150 mg) were homogenized in 400 μl of lysis buffer, along with ca. 50 grains of sterilized sea sand, using a pre-cooled glass mortar and pestle and processed for total soluble protein exactly as described previously (Jung et al. 2005). Equal amounts (200 μg) of protein were subjected to 2-DGE as described recently (Agrawal and Rakwal 2006). The 2-DGE [isoelectric focusing (IEF) in the first-dimension, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension] was performed by standard methods utilizing the hand-cast Nihon Eido 2-DGE system exactly as described (Jung et al. 2005; Agrawal and Rakwal 2006). Ca. 200 μg total protein extract was loaded onto the basic end of the IEF tube gel. A total of five 2-D gels were run using total soluble protein extracts prepared from KN and control; four gels were used for N-terminal amino acid sequencing after transfer onto polyvinyldifluoride (PVDF) membranes and Coomassie brilliant blue (CBB) staining, and one CBB stained gel was used for spot excision and subjected to MS.

Amino acid sequencing and homology search

N-terminal amino acid sequencing of PVDF protein spots was done as described previously (Jung et al. 2005). The N-terminal amino acid sequences obtained were used to interrogate databases with Web-accessible search programs like Fasta3, available online from EMBL Outstation of the European Bioinformatics Institute, and the FastA to identify homology to proteins already present in the protein and nucleic acid databases.

Mass spectrometry

The protein spots were excised from the CBB-stained 2-D gels, using a gel picker, and transferred to sterilized Eppendorf tubes. Gel pieces were incubated in 0.2 M NH_4HCO_3 (pH 7.8) for 20 min, shrunk by dehydration in acetonitrile, which was then removed, followed by washing with vortexing in the same volume of acetonitrile and 0.1 M NH_4HCO_3 (pH 7.8). The solution was removed, and the gel pieces were dehydrated with vortexing by addition of acetonitrile, and swelled by rehydration in 0.1 M NH_4HCO_3 (pH 7.8). The dehydration process was repeated twice, and the gel pieces were completely dried in a vacuum centrifuge. Gel pieces were swollen in a digestion buffer containing 10 μg/ml of trypsin (Promega, sequencing grade) on ice. The digestion buffer was removed after 45 min incubation and replaced with 20-30 μl of 50 mM NH_4HCO_3 (pH 7.8). The gel pieces were then incubated at 37 ℃ for 8-12 h. The supernatant was desalted through a C18 ZipTip (Millipore, Bedford, MA, USA) according to the manufacturer's protocols, and a 2-8 μl (minimum 1 pg by standard chemical; dilution of each sample, 1, 5, and 8 μl was analyzed) solution was injected for analysis with nano electrospray ionization-liquid chromatography-tandem mass spectrometry (nESI-MS/MS) (Agilent, Palo Alto, CA, USA). The nLC was performed with an Agilent 1100 NanoLC-1100 system combined with a micro well-plate sampler and thermostatted column compartment for preconcentration (LC Packings, Agilent). Samples were loaded on the column (Zorbax 300SB-C18, 150 mm × 75 μm, 3.5 μm) using a preconcentration step in a microprecolumn cartridge (Zorbax 300SB-C18, 5 mm × 300 μm, 5 μm). Four μl of the sample was loaded on the precolumn at a flow rate of 15 μl/min. After 5 min, the precolumn was connected with the separating column, and the gradient was started at 300 nl/min. The buffers used were 0.1% HCOOH in water (A) and 0.1% HCOOH in acetonitrile (B). A linear gradient from 2-70% B for 25 min was applied. A single run took 75 min, which included the regeneration step. A LC/MSD Trap XCT with a nano electrospary interface (Agilent) was used for MS. Ionization (2.0 kV ionization potential) was performed with a liquid junction and a noncoated capillary probe (New Objective, Cambridge, MA, USA).

For tandem mass spectrometry, peptide ions were analyzed by the data-dependent method to collect ion signals from the peptides in a full mass scan range (*m/z* 300-2200). After determination of the charge states of an ion on zoom scans, an MS/MS spectrum was recorded to confirm the sequence of the precursor ion using collision-induced dissociation (CID) with a relative collision energy dependant on molecular weight. The individual spectra from MS and MS/MS data were submitted to the Agilent Spectrum Mill MS proteomics workbench (Agilent, Palo Alto, CA, USA) for protein identification. Proteins were identified by database search against the Updating Databases SWISS-PROT (ftp://www.expasy.ch/databases/sp_tr_nrdb/fasta/sprot.fas.gz) and NCBI (ftp://ftp.ncbi. nlm.nih.gov/blast/db/FASTA/nr.gz) using default parameters. Modifications of methionine and cysteine,

peptide mass tolerance at 2 Da, MS/MS precursor mass tolerance 2.5 Da and product mass tolerance 0.7 Da, allowance of missed cleavage at 2, and charge states (+1, +2, and +3) were taken into account. For all protein assignments, a minimum of two unique peptides was required. Only significant hits as defined by the probability analysis were considered initially.

Results and Discussion

Our preliminary study had already reflected on a role for KN in rice plant self-defense mechanisms (Rakwal et al. 2003), thus taking forward the enquiry on cytokinins and plant defense a step forward. In our previous experience on the rice seedling leaf "*in vitro*" model system, to investigate the effect of different environmental factors, we had occasionally observed a difference in the response in rice leaf when exposed to either continuous light or dark (Agrawal et al. 2001). Knowing well that the light/dark effects may influence the response even in the case of KN, we decided to look at this aspect in the present study. For the experiment the KN-treated leaves were incubated under either continuous light or dark, and leaves were photographed for the differential symptoms observed therein. It was found that KN causes the clear formation of brown spots or lesion-like symptoms on the leaves, increasing in a time-dependent manner (Fig. 1). Contrastingly, under darkness, no such brown spots/lesions were observed on the leaf, strongly suggesting an effect of light in modulating KN-induced morphological symptoms on leaf.

The next question we asked was whether dark incubation can also effect the induction of two classes of molecular markers, namely the rice phytoalexins sakuranetin and momilactone A, and certain stress-related proteins. Both sakuranetin and momilactone A were strongly induced by KN treatment under light incubation, but not at all under darkness (Fig. 2). Interestingly, sakuranetin accumulation showed a time-dependent increase

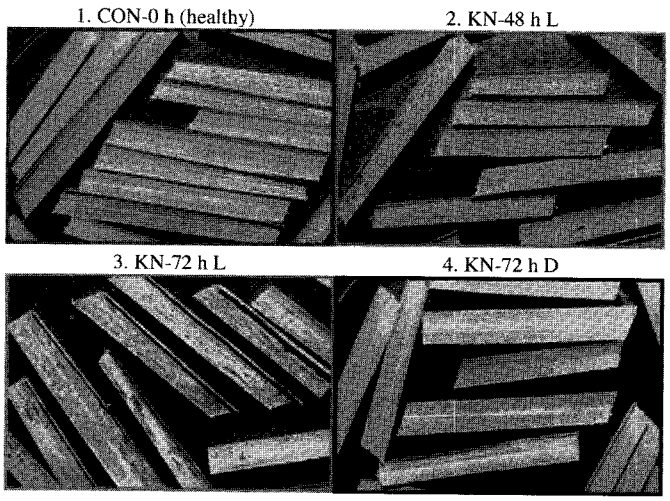

Fig. 1. Kinetin (KN, 100 μM) causes necrotic lesion formation and interveinal yellowing on detached rice seedling leaf segments. 1 to 4: 0 h (at the start of the experiment), 48 h, 72 h (continuous light) and 72 h (continuous dark).

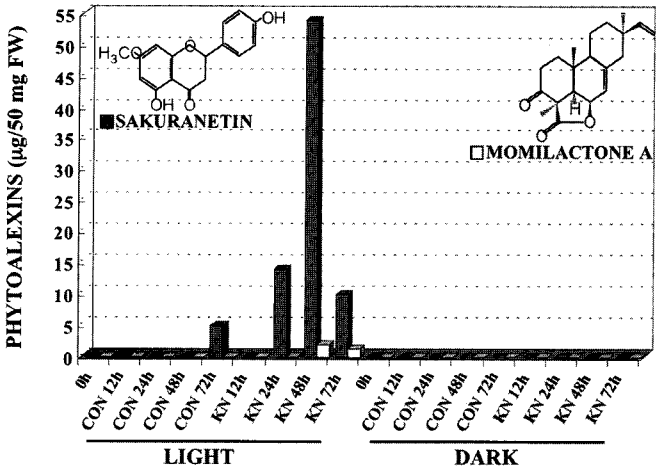

Fig. 2. Time-dependent accumulation of phytoalexins, sakuranetin (filled bars) and momilactone A (empty bars) in rice seedling leaf segments. Treatment was carried out under continuous light (left) or dark (right). CON, control (MQ water); KN, kinetin (100 μM). Sakuranetin and momilactone A were quantified by LC-MS/MS technique. Results are the means of two independent experiments.

peaking at 48 h after KN treatment followed by a dramatic decline at 72 h; control leaves also showed a small increase in sakuranetin level only at 72 h after treatment. On the other hand, momilactone A was first detected at 48 h after treatment and declined to a low level at 72 h; no momilactone A was detected in the controls. These results clearly demonstrate a link between light and KN-induced rice phytoalexins.

Finally, we looked at the protein profiles in leaves after KN treatment under both light and dark using 2-DGE (Figs. 3 and 4). KN treatment induced numerous changes in protein spot patterns in a time-dependent manner, and 11 proteins spots which were reproducibly detected are marked in Fig. 3. The corresponding controls showed almost no change. Interestingly, the dark incubated and KN-treated leaf segment protein profile was almost similar to the control except for a few spots which were repressed. These protein spots were either N-terminal sequenced (numbered as Arabic numerals) after blotting the separated proteins on gel onto a PVDF membrane stained with silver or taken for MS

Table 1. Amino acid sequence and homology of rice leaf proteins identified by Edman sequencing.

Spot No	MW (kDa)	Sequence	Homologous Protein	Accession Number	Homology (%)
1	17	N-APVSISDERA	OsPR10a (rice)	Q9LKJ8	70.00
2	18	N-APXVSDEHAVAVS AERLWKAFMDASTLPK	OsPR10b (rice)	Q9LKJ9	90.00
3	19	N-ATFTINRXS	Thaumatin protein precursor (OsPR, rice)	P31110	90.00
4	49	N-blocked; I-TGEIKGX	RuBisCO LSU	P30828	90.00
5	27	N-LSAKNYFMAXYEP	RuBisCO LSU	AAG09793	75.00
6	26	N-LSAKNYGRAX	RuBisCO LSU	O63190	100.00
7	25	N-SFYFNAEAIY	RuBisCO LSU	Q9XR03	89.00
8	23	N-TLTGGFTANT	RuBisCO LSU	P92310	100.00
9	20	N-TKETETKDTDVL	RuBisCO LSU	P31195	75.00
10	15	N-FQVWPIEGIK	RuBisCO LSU	P05347	100.00
11	14	N-RARGIFFTQD	RuBisCO LSU	P12089	100.00

Table 2. Rice leaf proteins and their identification by MS.

Spot No.	Protein Name	%AA cover-age	Distinct Peptides	Distinct summed MS/MS Search Score	Swiss-Prot/NCBI* Accession	P/I MW (Da)
a	Ribulose bisphosphate carboxylase large chain precursor	16	7	97.44	P12089	6.22/52881
b	ATP synthase beta chain	65	23	384.06	P12085	5.47/54014
c	Fructose-bisphosphate aldolase, chloroplast precursor	18	7	105.91	Q40677	7.59/42148
d	Ribulose bisphosphate carboxylase large chain precursor	18	8	115.58	P12089	6.22/52881
e	Ribulose bisphosphate carboxylase large chain precursor	15	8	121	P12089	6.22/52881
f	Ribulose bisphosphate carboxylase large chain precursor	9	4	60.95	P12089	6.22/52881
g	Pathigenesis -related protein PR -10a	40	6	100.65	9230755	4.96/16657

(marked alphabetically) after spot excision from CBB-stained gel. The N-terminal amino acid sequences and MS data analysis results are presented in Tables 1 and 2, respectively. Prominent among the KN-affected proteins were ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large and small subunits (LSU and SSU; spots 4/a, and 10) which were strongly down-regulated after treatment with KN under light over corresponding controls. These two proteins did not significantly change under darkness and KN treatment. Both the RuBisCO subunits have

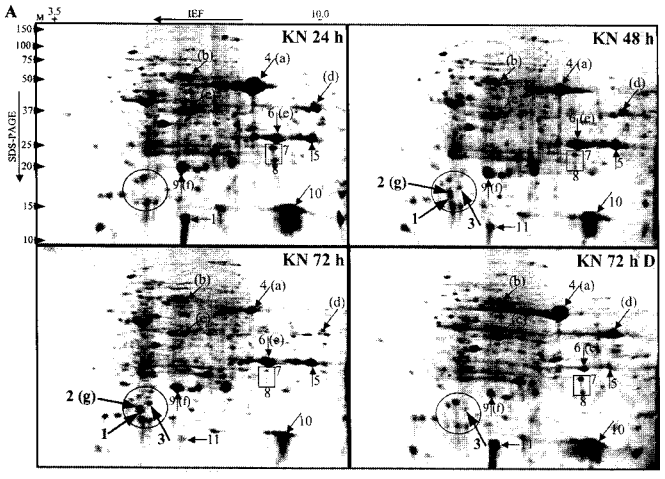

Fig. 3. Representative 2-D gels of rice leaf proteins changed by kinetin (KN, 100 μM). Proteins (200 μg equal amounts for each sample) were separated in the first dimension (pH 3-10) and second dimension (SDS-PAGE 15%). KN-treated rice leaves were sampled at 24, 48 and 72 h after treatment under continuous light (L) or at 72 alone under continuous dark (D). The significantly changed protein spots are marked and numbered (1-11, used for N-terminal amino acid sequence; and a-g, used for MS). The three protein spots in circles and marked by bold arrows indicate the marker PR protein family. Seven μls of the molecular mass standards (Precision Plus Protein Standards) were loaded in the well or next to the acidic end of the IEF tube gel. Electrophoresis was carried out at a constant current of 35 mA for 2-1/2 h or until the dye (250 μL BPB; 0.1% (w/v) in 10% (v/v) glycerol in MQ) reached the bottom of the gel (for details see Agrawal and Rakwal 2006).

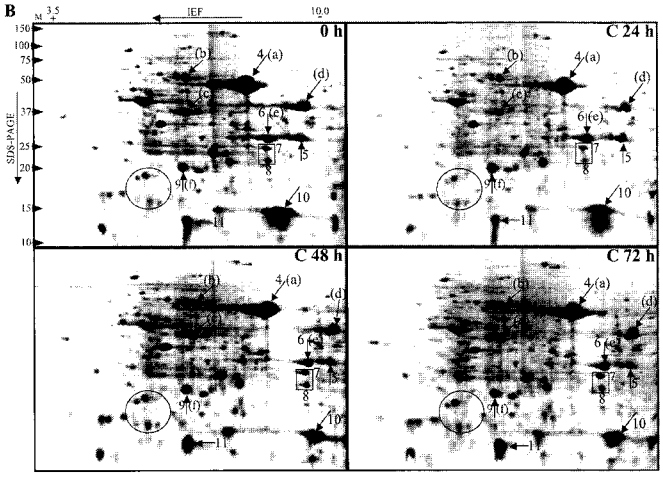

Fig. 4. Representative 2-D gels of rice leaf proteins in the control experiment. 0 h, healthy cut leaf segments. 2-DGE was carried out as described in Fig. 3. For reference, the protein spots as marked for identifying KN-induced changes are also marked and numbered (1-11, used for N-terminal amino acid sequence; and a-g, used for MS). The circles indicate the same position of the three protein spots induced by KN in Fig. 3.

been shown to be slightly or dramatically reduced in the rice leaf, under various stresses, including the gaseous pollutant ozone, phytohormone jasmonic acid, and the fungal elicitor chitosan (Agrawal and Rakwal 2006). These results indicate that KN affects photosynthesis/energy metabolism, especially under light conditions. Moreover, protein spots 5-9 and 11, were also identified as RuBisCO or its fragments, and these in particular were reduced in KN-treated leaves prominently under darkness, suggesting that the RuBisCO protein turnover is being affected differentially under darkness and KN treatment. We also found two classes of pathogenesis-related (PR) proteins (for review see Jwa et al. 2006), namely OsPR10 (a,b, spots 1 and 2/g) and OsPR5 (spot 3), which were potently induced by KN under light, but not under darkness. The control leaves did not have these two protein families. Therefore, it can be suggested that light signals play an important role in KN-triggered accumulation of secondary metabolites and defense/stress-related proteins. In combination, the above findings reveal the differential behavior of rice leaves to KN under light/dark.

Conclusions

In conclusion, we demonstrate that the effect of KN on rice plant self-defense mechanism (accumulation of marker secondary metabolites and stress proteins) is highly dependent on light signals. Although in the present study we report the light regulation of KN responses, it is still not clear as to how incubation under dark prevents the KN-induced accumulation of secondary metabolites and proteins seen under light. We speculate that under dark incubation, KN may not be perceived as a signal, thus rice leaves show none of the molecular changes observed with KN treatment under light.

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