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Original Article

Protein Kinase C as a Biomarker for Assessing the Effect of Environmental Stress and Fungal Invasion on Plant Defense Mechanism

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Plants are known to activate specific defense mechanisms in response to invasion by pathogens and environmental stresses. We examined the possibility of utilizing the changes in the amounts of protein kinase C (PKC) and its associated components of this signal transduction pathway as a biomarker of exposure of plants to the stress factors, using young rice plants as a model and a Western blotting method as the experimental tool. Preliminary studies have shown that PKC is a consistently more sensitive marker of exposure to a variety of environmental stress factors as compared to phospholipase C (PLC) or G-protein. The titer of PKC increased as a result of exposure to herbicides, low concentrations of copper, fungicides and other chemicals. The same trend was observed when rice plants were stressed by severe physical treatment such as broken stems, deprivation of sunlight and transplantation. On the other hand, PKC levels decreased upon exposure to high winds and high concentration of copper. The most drastic rise in PKC was observed when rice plants were inoculated with the rice blast fungus *Pyricularia oryzae*. As expected, several plant protectants against the fungal invasion also induce a rise in PKC.

INTRODUCTION

In the environment, plants receive a variety of stresses, including changes in nutritional and climatic conditions, water qualities, exposure to pesticides, heavy metals, air and water pollutants, attacks by plant pathogens and insects, flooding, and drought *etc*. While in the past most of these effects have been studied in isolation mostly from the viewpoint of assessing the affected yield of the given crops, in the real world they seldom do act alone. Rather, they interact synergistically, antagonistically or in more complex manners. The key question we must ask then is how to assess the end results of such complex interactions on the given species.

In recent years a variety of approaches have been developed as biomarkers for assessing biological affects. Ideally such technologies should provide complementary data for exposure such as chemical residues which indicate the incidence of exposure but do not show the biological effects. Certainly what finally matters to the organisms is whether such exposure will cause an eventual stress or not. To be a useful marker, the criterion selected must be sensitive and responsive to environmental stimuli. Furthermore, if that biomarker is going to be used for the studies for multiple stresses it should react to many different types of stresses and moreover, the end result should last for some reasonable duration. Recently plant physiologists and pathologist have discovered that many plants respond to the invasion of pathogens by activating a set of defensive schemes such as overproduction of phenols, oxidative enzymes and calcium dependent protein kinases. Moreover, it is well known that such a defensive response could also be triggered by some organic chemicals alone, without actual presence of live pathogenic organisms. They are collectively called "elicitors".[1] The driving forces in turning on and maintaining this defensive response are known to be the protein kinase C/inositol triphosphate signal transducer.[2] It occurred to us, therefore, that some of the key components of this pathway might provide us

with reliable biomarkers indicating their state of stresses and the extent of plant activation in their defensive commitments.

The rice plant was chosen as the study species in view of the availability of fundamental technology and as well as their importance in providing staple food to large population. In addition, we examined effects of the chemical probenazole (PBZ), which was known to act as a protectant of rice plants against blast disease by priming the plants to preactivate their defense resistance in rice.[3-9]

MATERIALS AND METHODS

1. *Chemicals*

Probenazole (Oryzemate®, 3-allyloxy-1,2-benzisothiazole-1,1-dioxide) was provided by Meiji Seika Kaisha, Ltd., Japan. Other reagents used were of the highest grade commercially available.

2. *Plants and Fungus Inoculation*

Rice plant used was a cultivar Aichiasahi with a resistant genotype *Pi-a* to rice blast. Ten seeds were presoaked in water and after germination, sown in a pot (6.2 cm diameter, 8 cm height) with soil. Plants were grown at 25°C at 40–60% relative humidity in a greenhouse till they reached the 3–4 leaf stage.

Rice blast fungus, *Pyricularia oryzae* (Hoku-1 strain, race 007) was cultured, and spores (10^6/ml) were sprayed directly on rice leaves. Plants were kept in a chamber at 100% relative humidity for 24 hr and then transferred to a greenhouse. The effects of the treatments described below were determined by the observation of lesions developing on leaves within the 2 week time span.

3. *Environmental Stress*

The effects of environmental stresses were studied using 3–4 leaf stage plants grown in pots, each containing 10 plants as described above. Unless stated otherwise, each stress treatment was given to one half of one pot (*i.e.* 5 plants), the other half serving as untreated control for a side-by-side comparison. The effect of sunlight was studied in the greenhouse by exposing plants in one half of a pot to mid-day sunlight for 2 hr. At the same time the other half of the same pot was covered with a black bag to shield the plants from sunlight for the duration of the experiment. The effect of the time of the day was studied by harvesting one half of a pot in the greenhouse at 9 am in a sunny day and the other half at 9 pm (12 hr later, on the same day). The effect of wind exposure was studied by exposing one full pot with ten plants to strong wind generated by an electric fan (wind speed of 126 m/min and force of 8.6 m^3/min) in a mid-day time point for 1 hr. An equivalent, unexposed pot with rice plants grown at the same time for the same duration in the greenhouse served as control in this case.

For the study on the effect of the stem breakage, the stems of rice plants in one half of a pot were bent at approximately 10 cm above the ground level by hands so that their stems were clearly broken. Again, the other half of the same pot served as control. They were maintained for subsequent 24 hr in the greenhouse before harvest. The effect of water immersion was studied by submerging the full foliage part of rice plants in one half of a pot in distilled water at room temperature at 24°C for 1 hr by holding the pot upside down. The other half, also held upside down, but without immersion in water, served as control. The effect of transplanting was studied, first by soaking pots in water to soften the soil, then transferring plants in a large water container to completely loosen their roots, and subsequently repotting plants with washed roots into a new pot. The care was taken so as not to damage the roots during washing and transfer. After replanting, the plants were harvested after 24 hr. A control pot which received only the initial soaking treatment was also kept for 24 hr. All were kept in the greenhouse.

4. *Effects of Herbicide Paraquat and Copper in Rice Plants*

Aqueous solution of paraquat was prepared by diluting a commercial concentrate (Nihon Nohyaku, Gramoxone 24%) plus 1% wetting agent with distilled water to give 30 ppm concentration. Young plants in pots (38–42 cm high) were held in such an angle that all parts of their leaves were soaked in paraquat solution and held for 30 min at room temperature. Copper solution was prepared by dissolving $CuSO_4 \cdot 5H_2O$ in distilled water. The volume of water to give uniform wetting of the volume of soil in a standard pot, without causing excess dripping was predetermined and the amount of copper equivalent to give 1, 3, 10, 30 or 300 ppm was given to each pot with growing rice plants.

5. *Effects of Fungus Infection and a Chemical Plant Protectant PBZ in Rice Plants*

Application of PBZ to rice plants was performed essentially as described by Watanabe *et al.*[8] The 40 ml solution was prepared by dissolving PBZ into distilled water. The solution was poured onto the soil surface of each pot. The treated pots were kept moist. The administration of the PBZ treatment in drench, submerged application or root immersion was performed 5 days before the rice plants were inoculated with spores of the pathogen, *P. oryzae*.

6. *Extraction of Rice Plant Protein*

Rice foliage was obtained by cutting plants at approximately 1 cm above the soil. They were weighted, quickly frozen in liquid nitrogen, cut into small pieces and pulverized using a mortar and pestle. The extraction

was performed by the method of Gegenheimer.[10] The samples were stored at −80°C until Western blotting analysis.

7. Western Blotting

Total protein was determined by using BioRad reagent (Bio-Rad Laboratory Ltd.). Forty micrograms of protein loaded into each well of a mini-gel and electrophoresed on a 10% SDS polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk and then incubated with the primary antibody in 1% bovine serum albumin in TBST buffer (10 mM Tris-HCl, pH 8.0, containing 150 mM sodium chloride and 0.06% Tween 20) overnight at 4°C. After washing 3 times with TBST buffer, the HRP secondary antibody (horseradish peroxidase-labeled antibody) was added (1:7500), incubated for 1 hr at room temperature, washed 3 times and the bands were visualized using ECL Western blotting chemiluminescent detection reagents (Amersham Life Science, Arlington Heights, USA). We used primary antibodies for G-protein, PLC and PKC; anti-G-protein α-subunit internal (40-54) was purchased from Calbiochem-Novabiochem Corporation (rabbit source) and anti-PLCγ1 (1249) and anti-PKC (MC5) were from Santa Cruz Biotechnology, Inc. (rabbit polyclonal and mouse monoclonal source, respectively).

RESULTS

1. Components Search as Potential Biomarkers

A simplified scheme of plant defense signaling pathway is shown in Fig. 1. Three of its essential components have been tested for their suitability as potential biomarkers. Examples of each Western blotting ECL data are shown in Fig. 2. Two of the antibodies, one to PKC and the other to G-proteins, detected only one major immunoreactive protein band: 85 kDa protein for PKC and 55 kDa protein for G-protein. In the case of PKC, the molecular weight value matched well with the published data.[11] On the other hand, the G-protein detected here did not match with the known molecular weight of the GTP binding, Gα-subunit (44.5 kDa).[12,13] A preliminary study using bovine Gα-protein showed clearly that this antibody reacts with the 39 kDa, bovine Gα-protein (data not shown). Thus, the absence of such a band corresponding to the 39 kDa protein in rice plant extracts indicates that either Gα-proteins is not present in this material at a sufficient level large enough to be detected by this method, or the corresponding rice Gα-protein does not have quite the same amino acid sequence to be detected by this antibody. In contrast, the antibody to PLC initially reacted with several nonspecific protein bands in addition to one specific

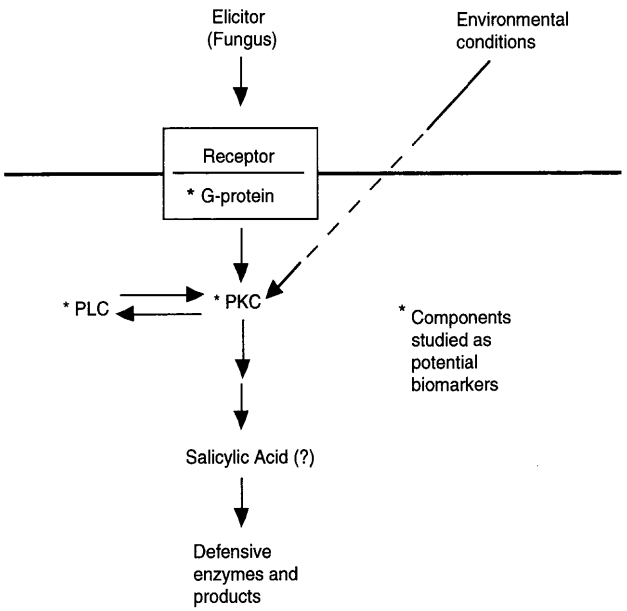

Fig. 1 Scheme of plant defense mechanism.

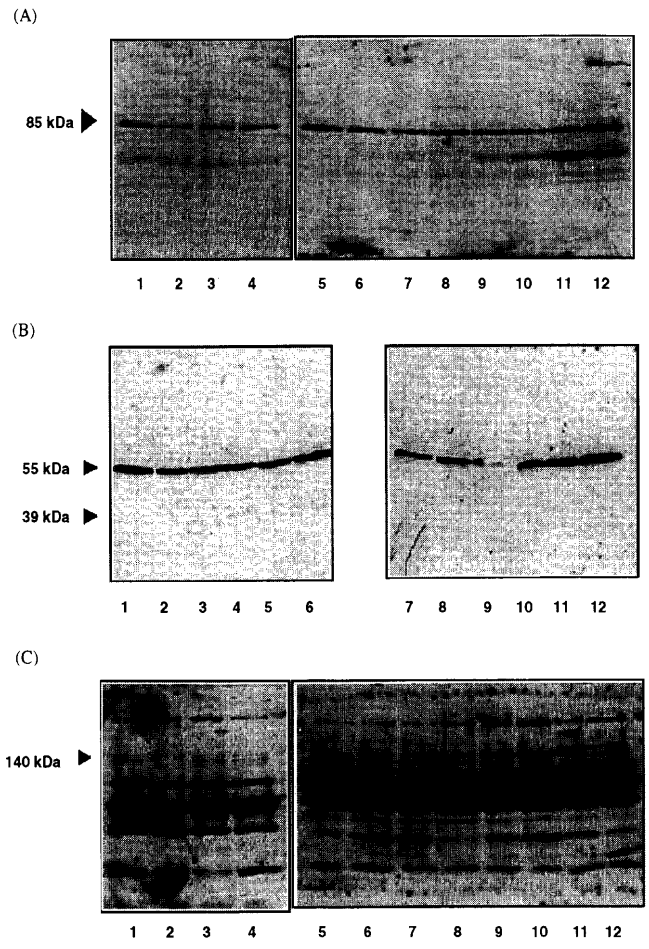

Fig. 2 Effects of changes in various environmental conditions on the titers of protein kinase C (PKC), G-protein and phospholipase C (PLC) in rice plants.

The titers were assayed using Western blotting. (A) the titer of PKC, (B) the titer of G-protein, (C) the titer of PLC: Lane (1) sunshine, (2) cloudy, (3) day time, (4) night, (5) no wind, (6) wind, (7) stem unbroken, (8) stem broken, (9) normal, (10) immersed in water, (11) undisturbed, (12) transplanted.

Table 1 Effects of change in various environmental conditions on the titer of PKC, G-protein and PLC in rice plants.

		PKC	G-protein	PLC
Sunlight	Sunshine	100± 0.5	100± 5.5	100± 5.7
	Cloudy	67± 1.1	113± 1.4	92± 7.6
	Day time	100± 0.7	100± 9.6	100± 9.8
	Night	85± 2.8	96± 8.1	30± 4.8
Windy		58± 1.0	91± 2.0	174±19.0
Stem broken		224± 1.0	183±13.0	442±21.0
Immersed in water		122± 6.0	84± 6.0	83±10.0
Transplanted		162± 7.0	93± 7.0	130± 2.0

The titers of protein kinase C (PKC), G-protein and phospholipase C (PLC) were assayed using Western blotting. The value of cloudy and night showed % against sunshine and day time, respectively. The others were shown % value against undisturbed. Data represent the means±SE of four replicates.

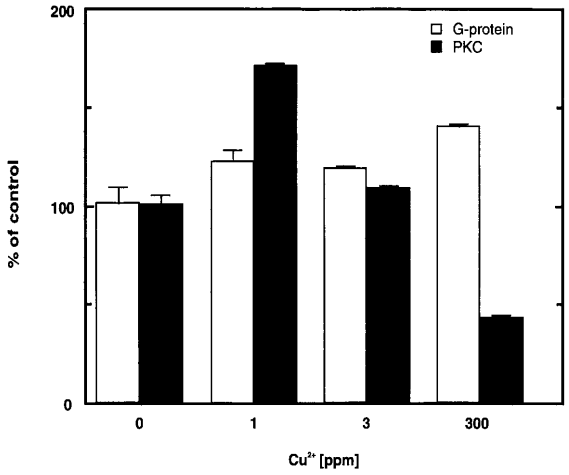

Fig. 3 Effects of $CuSO_4$ on titers of G-protein and PKC.

The titers of G-protein and protein kinase C (PKC) were assayed using Western blotting. The values were shown by % against no treatment. Means and standard errors are based on data from three replicates.

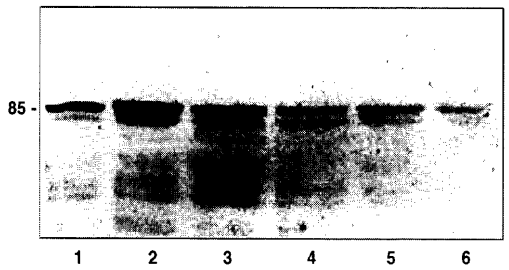

Fig. 4 Effects of herbicide paraquat, $CuSO_4$ and then combined effects with heat treatment on protein kinase C in rice plants as detected by Western blotting using an antibody to protein kinase C.

Lane (1) control, (2) soaking in 30 ppm paraquat 30 min, and tested after 24 hr, (3) 30 ppm of $CuSO_4$ applied to soil (24 hr), (4) high temperature treatment 40°C (1 hr), (5) paraquat treatment as above and then heat treatment, (6) $CuSO_4$ treatment as above then heat treatment.

protein band at the expected molecular weight (140 kDa). However, fortunately the location of this specific band was such that other nonspecific proteins did not cause overlapping or misinterpreting problems. Thus, with careful blocking and the use of a lower amount of the primary antibody and parallel testing with irrelevant antibody preparations, we could readily reorganize the PLC band based on the data reported.[14-16]

The summary of various treatment test results is shown in Table 1. The titer of PKC was found to readily respond to changes in various environmental conditions. The lack of sunlight and windy conditions lowered its titer, whereas stimuli such as breaking of stems and transplantation induced the rise. In contrast, the responses of the titer of G-protein and PLC were not so sensitive, though breaking of stems consistently elevated all of these components. Further, when the effects of copper ions on G-protein and PKC were examined, the latter responded well to induce the rise at low concentration but significantly to reduce at high concentration (Fig. 3).

2. *Effects of Herbicide Paraquat and Copper*

Combined effects of heat (40°C) and herbicide paraquat or heat and copper treatment were studied separately. The result (Fig. 4) showed that both paraquat and copper increased the titer of PKC while heat treatment caused its decrease. The combined effect was significant reduction in the amount of PKC in plants, the most severe case being the effect of heat and copper.

3. *Effects of Fungal Infection and Systemic Application of PBZ*

To verify that the system studied here represents plant defense response, we next tested the effects of infections by the fungus and treatment of the chemical probenazole (PBZ), which is also known to induce the defense capability. The treatment of PBZ with various concentra-

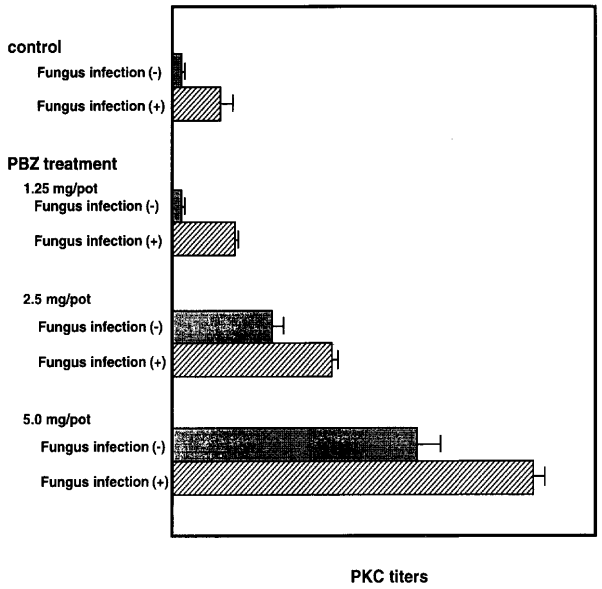

Fig. 5 Effects of fungus infection and a chemical plant protectant PBZ.

The titer of protein kinase C (PKC) were assayed using Western blotting. Probenazole (PBZ) treatment was performed with various concentrations before fungus inoculation. Means and standard errors are based on data from three replicates.

tions was performed 5 days before the inoculation of the rice plants with spores of the pathogen *P. oryzae*. Two days later, we examined the titer of PKC (Fig. 5). One can clearly see that the fungus infection causes a rise in PKC titer. However, the effect of the treatment with PBZ alone was also significant at the application rates higher than 2.5 mg/pot. Thus, the PBZ treatment before fungus infection raised the titer of PKC.

DISCUSSION

By using Western blotting we were able to measure, in the whole foliage, the total titer of proteins that are antigenically reacting with the primary antibody and are matching with the expected molecular weight range. It must be mentioned that titers of this types of signal transduction proteins do not automatically indicate the functional activity levels as they are often modulated further through phosphorylation, translocation within cells to active sites, assemblage with other signal transduction components *etc*. Nevertheless, titers of these proteins are related generally to the levels of mRNA, and therefore their changes indicate the balance of transcriptional and translational activities against mRNA degradation. Therefore, what we have assessed in this study are likely the responses of rice plants to those external stimuli with respect to changes in these protein levels through modification of the rates of mRNA synthesis and degradation.

We could show that the rising levels of PKC represent the activation of plant defense systems by demonstrating that PKC levels increase as a result of fungus infections, and treatment by plant protectant. We also found that PKC was very responsive to certain environmental stimuli such as transplantation and broken stems. On the other hand, its levels were not always elevated by stresses, or responsive to all stimuli. Therefore, PKC should not be regarded as a universal marker for all environmental stresses.

Nevertheless, PKC could be an excellent biomarker as long as its modulatory principles are understood. Clearly, the rise in PKC is an "induction" phenomenon, occurring in healthy individuals responding to external threats. Thus, stress conditions which overwhelm the health of plants such as exposure to very high concentration of copper, or extreme dehydration, would compromise the ability of plants to augment their defensive capabilities. Moreover, there appear to be some physiological conditions that do not promote the full defensive responses. A good example is the low PKC level at night and high humidity, two conditions known to be most favorable to fungus invasion of rice plants. Therefore, PKC appears to be an excellent biomarker as long as the above principles and some limitations such as optimum operative ranges are clearly understood. It is basically a marker for "plant defense" ability and activity.

The main use of PKC as a biomarker should be for studies on the effects of chemical and physical stressors on plant's defense activities and capabilities. For the studies on the effects of chemical, PBZ was used. PBZ is an effective agricultural chemical for managing rice blast disease. Rice plants treated with PBZ show defense response following the inoculation of compatible rice blast fungus. PBZ would thus appear to induce disease resistance in rice. In fact, the treatment of PBZ caused a rise in PKC titer, and the fungus infection with PBZ treatment for 5 days caused a high level of induction of PKC. Many plants can respond to pathogen infection by inducing long-lasting, broad-spectrum resistance. This phenomenon, referred to as systemic acquired resistance (SAR), has been studied for many years,[17,18] but the biochemical events leading to maintenance of an induced-resistance state are not clear. Although SAR has been described in many plant species and seems to be ubiquitous, it has been most extensively studied at the biochemical level in tobacco, cucumber and *Arabidopsis*.[19,20] In tobacco, salicylic acid (SA) accumulation correlated with the SAR-related gene products.[21] While whether SA acts as a long-distance systemic signal for SAR induction still remains unclear in monocotyledonous plants. Our preliminary study suggested that PBZ could induce the rise in SA and its β-glucoside in the plants (unpublished data). Since the proposed active metabolite of PBZ, BIT (1,2-benzisothiazol-3-(2*H*)-one-1,1-dioxide), was shown to

enhance the potential plant defence abilities through activation of GTPase in plasma membrane prepared from rice leaves,[22] PBZ may act as a priming effector in earlier step(s) of signal transduction pathway to induce the rise of PKC and SA level in plant cells.

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要　　約

植物の環境ストレスと病原菌感染における防御機構のバイオマーカーとしてのプロテインキナーゼC

桐渕協子，Debra Y. DUNLAP，Fumio MATSUMURA，山口　勇

植物には，環境ストレスや病原菌感染に対する独自の抵抗性機構が備えられていると考えられている．我々は，情報伝達系物質であるGタンパク質，フォスフォリパーゼC（PLC），プロテインキナーゼC（PKC）の量変化を調べることにより，環境ストレスに対するバイオマーカーとしてのテストを行なった．モデル植物としてイネを使用し，ウエスタンブロッテング法を用いた．その結果，様々な環境ストレスに対してPKCが最も敏感に反応し，また感受性も非常に高いバイオマーカーであることが判明した．PKCの量は除草剤，低濃度の銅イオンの存在により増加した．また，茎を折るなどの物理的処理をした場合においても同様の傾向がみられた．一方，強風や高濃度の銅イオンではPKCレベルの減少がみられた．PKCレベルは，病原菌（いもち病菌）をイネに接種した時に顕著に増加した．また，いもち病に対する非殺菌性防除剤であるプロベナゾール（PBZ）を処理した場合もPKCレベルは増加した．