Epidermal Growth Factor Binding Proteins in Rice (*Oryza sativa* L.) Leaves

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A ligand blotting experiment using [125 I]epidermal growth factor (EGF) showed that there are EGF binding proteins in rice leaves. EGF binding proteins have isoelectric points and relative molecular masses of 5.5/35 kDa, 7.5/50 kDa and 8.3/40 kDa, respectively. The amino acid sequences of the N-terminal and internal regions of the EGF binding proteins were determined. The amino acid sequences of 35 kDa protein and 40 kDa protein were found to be homologous with those of the photosystem II oxygen-evolving complex. The amino acid sequence of the 50 kDa protein was homologous with that of ribulose biphosphate carboxylase/oxygenase. The presence of proteins capable of binding to EGF suggests the possibility of EGF-like regulation in plants.

Key words rice; epidermal growth factor; leaf; binding protein

Herbivory, microbial infection, and mechanical wounding all induce the signal transduction pathway, so that plants recognize and respond to environmental stress. Exactly how this recognition occurs and what response ensues have been the subject of various studies.¹⁾

The biochemical effects of herbivory, particularly evolutionary processes responsible for chemical defense systems in plants have been examined.^{1,2)} However, in addition to the negative-feedback response most often considered in such work, Dyer³⁾ and Dyer *et al.*⁴⁾ describe nonlinear growth involving positive- and negative-feedback from the response of *Sorghum bicolor* var. Rio seedlings to mouse epidermal growth factor (EGF). They suggest that plants may recognize and respond to animal biochemical messengers in the saliva of many herbivores. Human recombinant EGF stimulates maize (*Zea mays*) coleoptile growth,⁵⁾ promotes plant cell division in carrots (*Daucus carota*) and increases adventitious root formation in Azuki beans (*Vigna angularis*).⁶⁾

Other mechanisms of animal induction in plant-animal interactions may involve electrical signaling *via* chemical gradients,⁷⁾ transmembrane receptors to hormones,⁸⁾ production of peroxides in response to elicitors,⁹⁾ pectin polysaccharides^{10,11)} and the systemic release of chemical signals by herbivore-injured plants.^{12,13)} Interaction between EGF and indole-3-acetic acid may allow plants to recognize and respond to animal biochemical messengers with a consequent change in plant cell elongation and, ultimately, altered plant growth patterns.¹⁴⁾ In this regard, no one has attempted to integrate signal transduction literature into one common paradigm. In this study, an attempt is made to detect EGF-binding proteins by ligand binding assay. The plant photosystem II oxygen-evolving complex and ribulose biphosphate carboxylase/oxygenase are shown to express EGF binding activity.

MATERIALS AND METHODS

Plant Materials Rice (*Oryza sativa* L. cultivar Nipponbare) was used in this study. Rice plants were grown in vessels containing commercial soils blended with

chemical fertilizer and cultivated in an air-controlled greenhouse under natural light. The developing leaves from field-grown plants were collected.

Preparation of Protein Extract A portion (250 mg) of the 3rd leaves was removed and homogenized with 1 ml extraction buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 5 μ M sodium vanadate and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 15000 rpm for 5 min in a TMA-4 rotor (Tomy, Tokyo). The supernatant was used as the rice leaf extract.

Detection of Epidermal Growth Factor Binding Proteins Proteins capable of binding to [125 I]EGF (Amersham, Buckinghamshire) were detected by a ligand binding assay.¹⁵⁾ Rice leaf extracts (1 mg protein) were separated by two dimensional-polyacrylamide gel electrophoresis (2D-PAGE)¹⁶⁾ and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. After transferring proteins to the PVDF membrane, it was dried at 37 °C for 5 min. This was followed by soaking at 4 °C; firstly, for 30 min, in saline (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.5 mg/ml sodium azide) containing 3% Nonidet P-40; secondly, for 2 h in saline containing 1% bovine serum albumin (BSA); and finally, for 10 min in saline containing 0.1% Tween 20. The PVDF membrane was then sealed in a plastic bag with 400000 cpm [125 I]EGF with 3 ml saline containing 1% BSA and 0.1% Tween 20. The PVDF membrane was incubated overnight at 4 °C. The PVDF membrane was then washed twice for 15 min at 4 °C in saline containing 0.1% Tween 20, and then 3 times for 15 min in saline. The blot was dried and exposed to X-ray film for 7 d at -70 °C.

Competition Binding Assay EGF binding proteins electroblotted onto the PVDF membrane were incubated for 30 min at 4 °C with increasing concentrations of EGF and then the amount of [125 I]EGF bound to protein was measured. The total protein concentration in the rice leaf extract was assayed using a standard protein assay kit (Sigma, St. Louis).

Isolation of Proteins Capable of Binding to Epidermal Growth Factor Rice EGF-binding proteins were separated by 2D-PAGE and electroeluted. After this, the

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protein solution was dialyzed against water for 2 d and dried.

N-Terminal and Internal Amino Acid Sequence Analysis

The collected proteins were electroblotted onto the PVDF membrane and subjected to amino acid sequence analysis.¹⁷⁾ The internal amino acid sequences of the protein were determined as described by Cleveland *et al.*¹⁸⁾ The amino acid sequence analysis of proteins on the PVDF membrane was performed by a gas-phase protein sequencer (477A, Applied Biosystems, CA).¹⁹⁾

RESULTS AND DISCUSSION

Western ligand blotting as described by Komatsu and Hirano¹⁵⁾ has been used to detect insulin-binding protein in plants. Using this technique, rice leaf protein extracts (1 mg protein) were separated by 2D-PAGE and electroblotted onto a PVDF membrane. The blotted proteins were reacted with [¹²⁵I]EGF. Three proteins were able to bind with EGF (Fig. 1). EGF binding proteins have isoelectric points and relative molecular masses of 5.5/35 kDa, 7.0/50 kDa and 8.3/40 kDa, respectively. When rice leaf protein extracts (10 mg protein) were separated by 2D-PAGE, three proteins were also able to bind with EGF (data not shown).

Fig. 1. Detection of Proteins Bound to EGF in Rice Leaves

Western ligand blotting was performed as described in Materials and Methods. Right to left: isoelectric focusing for first dimension, top to bottom: SDS-PAGE for second dimension. (A) Proteins detected with Coomassie Brilliant blue. (B) Radioactive proteins detected with [¹²⁵I]EGF. Arrowhead shows the position of EGF binding proteins.

Fig. 2. Detection of Proteins Bound to EGF in Rice Leaves

(A) Radioactive proteins detected with [¹²⁵I]EGF. (B) Radio active proteins detected with [¹²⁵I]EGF containing 1 ng/ml unlabeled EGF. (C) Radioactive proteins detected with [¹²⁵I] EGF containing 10 ng/ml unlabeled EGF. (D) Radioactive proteins detected with [¹²⁵I]EGF containing 100 ng/ml unlabeled EGF. Arrowhead shows the position of EGF binding proteins.

Fig. 3. The Competitive Binding Assay

The triangles, squares and circles indicate spot 1, 2 and 3, respectively. The results are the mean values of triplicate assays.

The contribution of nonspecific binding was determined by measuring [125 I]EGF binding in the presence of excess unlabeled EGF (1, 10 and 100 ng/ml) to saturate specific binding sites. However, no nonspecific binding was found on the PVDF membrane (Fig. 2). The preliminary competitive binding assay showed specific binding at different affinities for spot 1 (35 kDa protein), spot 2 (50 kDa protein) and spot 3 (40 kDa protein) (Fig. 3).

The leaf proteins were separated by 2D-PAGE and the proteins were electroeluted from the gel. After electroelution, the protein was digested with *Staphylococcus aureus* V8 protease on SDS-PAGE gel and the internal amino acid sequences (Fig. 4) determined. In spot 1 protein, sequences of 20 amino acid residues were determined and a structural homology search indicated the amino acid sequence of the protein to be homologous with that of the photosystem II oxygen-evolving complex. In spot 2 protein, the sequences of 14 amino acid residues were determined and a structural homology search indicated the amino acid sequence of the protein to be homologous with that of the ribulose biphosphate carboxylase/oxy-

Spot	pI	MW (kDa)	Sequence	Homologous protein
1	5.5	35	ESFGGPFLVPSYXGSSFLDPXG : : : : : : : : : : : : : : -ESFGGPFLVPSYRGSSFLDPKG--	photosystem II oxygen-evolving complex 140
2	7.0	50	EXLRGGLDFTKDDEN : : : : : : : : : : : : : : -ECLRGGGLDFTKDDEN-----	ribulose biphosphate carboxylase/oxygenase 157
3	8.3	40	EGVPRRLTFDE : : : : : : : : : : : : : : EGVPRRLTFDE----- ESFGGPFLVPSYXGSS : : : : : : : : : : : : : : -ESFGGPFLVPSYRGSS----- ESVQPSDSDLGA : : : : : : : : : : : : : : -ESVQPSDSDLGA-----	photosystem II oxygen-evolving complex 1 photosystem II oxygen-evolving complex 140 photosystem II oxygen-evolving complex 219

Fig. 4. Amino Acid Sequence Homology between Rice Leaf EGF Binding Proteins and Homologous Proteins

Numbers indicate the amino acid residue number of each polypeptide. "X" shows that amino acid sequences of the protein were not determined under these conditions.

genase. In spot 3 protein, three major fragments of the protein were obtained: one from the N-terminal region, the others from the internal regions of the protein. In all, the sequences of 38 amino acid residues were determined and a structural homology search indicated the amino acid sequence of the protein to be homologous with that of the photosystem II oxygen-evolving complex.

However, there is no evidence that the function of these three proteins is similar to that of the EGF receptor. The structural characteristics of these three proteins were compared with those of the EGF receptor. No homology in the amino acid sequence was found among these proteins.

Some proteins that regulate biological responses in animal cells are also present in plants. Kato *et al.*^{5,6)} suggest that an EGF-like protein regulates various physiological response in plant cells. Higo *et al.*²⁰⁾ showed the human EGF gene to be incorporated in plant chromosomes, transcribed and translated. Such research may help clarify the characteristics and physiological mechanisms of EGF.

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REFERENCES

- 1) Fritz R. W., Simms E. L. (ed.), "Plant Resistance to Herbivores and Pathogens: Ecology, Evolution, and Genetics," University of Chicago Press, Chicago, 1992, p. 3.
- 2) Spencer K. C. (ed.), "Chemical Mediation of Coevolution," Academic Press Inc., San Diego, 1988.
- 3) Dyer M. I., *Proc. Natl. Acad. Sci., U.S.A.*, **77**, 4836 (1980).
- 4) Dyer M. I., Detling J. K., Coleman D. C., Hilbert D. W., "In Grasses and Grasslands," ed. by Estes J. R., Tyrl R. J., Brunken J. N., University of Oklahoma Press, Norman, Oklahoma, 1982, pp. 255—295.
- 5) Kato R., Itagaki K., Uchida K., Shinomura T. H., Harada Y., *Plant Cell Physiol.*, **32**, 917 (1991).
- 6) Kato R., Nagayama E., Suzuki T., Uchida K., Shinomura T. H., Harada Y., *Plant Cell Physiol.*, **34**, 789 (1993).
- 7) Wildon D. C., Thain J. F., Minchin P. E. H., Butt I. R., Reilly A. J., Skipper Y. D., Doherty H. M., O'Donnell P. J., Bowles D. J., *Nature (London)*, **360**, 62 (1992).
- 8) Bottger M., Hilgendorf F., *Plant Physiol.*, **86**, 1038 (1988).
- 9) Apostol I., Heinsteins P. F., Low P. S., *Plant Physiol.*, **90**, 109 (1989).
- 10) Farmer E. E., Ryan C. A., *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 7713 (1990).
- 11) Pearce G., Strydom D., Johnson S., Ryan C. A., *Science*, **253**, 895 (1991).
- 12) Tumlinson J. H., Lewis W. J., Vet L. E. M., *Scientific American*, **93**, 100 (1993).
- 13) Turlings T. C. J., MaCall P. J., Alborn H. T., Tumlinson J. H., *J. Chem. Ecol.*, **19**, 411 (1993).
- 14) Moon A. M., Dyer M. I., Brown M. R., Crossley D. A., Jr., *Plant Cell Physiol.*, **35**, 1173 (1994).
- 15) Komatsu S., Hirano H., *FEBS Lett.*, **294**, 210 (1991).
- 16) O'Farrell P. F., *J. Biol. Chem.*, **250**, 4007 (1975).
- 17) Hirano H., *J. Protein Chem.*, **8**, 115 (1989).
- 18) Cleveland D. W., Fischer S. G., Kirschner M. W., Laemmli U. K., *J. Biol. Chem.*, **252**, 1102 (1977).
- 19) Hirano H., Watanabe T., *Electrophoresis*, **11**, 573 (1990).
- 20) Higo K., Saito Y., Higo H., *Biosci. Biotech. Biochem.*, **57**, 1477 (1993).