# Comparative Study of Pollen Proteins of Rice by Isoelectric Focusing and High-Performance Liquid Chromatography

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**ABSTRACT**

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Pollen proteins of 22 rice cultivars (*Oryza sativa* L.) belonging to japonica and indica types were compared. The isoelectric focusing (IEF) band patterns of these two types of rice differed when analyzed in polyacrylamide gels. Different cultivars of the same type showed variations in peak patterns after high-performance liquid chromatography. These results showed that cultivars with a common ancestor have a similar peak pattern. The coefficients calculated on the basis of similarity of peak patterns of chromatograms were significantly related to the extent of match in genetic identity among cultivars. The IEF band pattern showed an independence of sampling time and location of growth. The results indicated that pollen proteins from living plants can be used for systematic and phylogenetic studies of rice cultivars.

The agronomic, morphological, and biochemical properties of rice plants have been used to identify rice cultivars. On the basis of these characteristics, Chinese rice has been differentiated into two main types: japonica and indica. The identification of rice cultivars is sometimes very difficult because of a rather complicated heterogeneity (Huang 1987) in the genotype. This heterogeneity is regarded as the result of interline breeding and the use of exotic germ plasms. In recent years, the differentiation of cereal cultivars has been successful using electrophoresis (Lookhart et al 1982, 1983; Sarkar and Bose, 1984; Ramirez and Pisabarro 1985; Guo et al 1986; Kazemie and Bushuk 1990), high-performance liquid chromatography (HPLC) (Bietz et al 1984, Marchylo and Kruger 1984, Lookhart et al 1987, Scanlon et al 1989, Huebner et al 1990), or the combination of both processes (Lookhart 1985, Lookhart and Pomeranz 1985, Lookhart et al 1989).

In most cases, the storage protein was used as the base material for cultivar differentiation (Glaszmann 1986). Based on studies comparing other plants, Krattinger et al (1979) and Petersen (1983) proved that pollen proteins from plants can be used for species or hybrid identification. However, no study has used pollen protein for the identification of plants under the species level. In Taiwan, different cultivars of rice have been bred, and their phylogenetic relationships are already well known. It is thus a new attempt in this paper using these cultivars by IEF and HPLC analyses in order to test the utility of pollen proteins for cultivar differentiation.

## MATERIALS AND METHODS

Pollen grains of 20 cultivars of japonica type and two cultivars of indica type rice (*Oryza sativa* L.) from different growing locations (Table I) were used for this study. The freshly collected pollen grains were refrigerated to avoid dehydration. The materials were preserved by plunging the pollen into liquid nitrogen and then storing it at −20° C.

**TABLE I**
**Cultivars of Japonica and Indica Rices Used for Sampling Pollen**

| Cultivar | Abbreviation | Locality[a] | Collection Date (1988) |
|---|---|---|---|
| Japonica | | | |
| Taipei 309 | TP 309 | NTU | June 30 |
| Hsinchu 64 | SZ 64 | NTU | July 2 |
| Taichung 65 | TC 65 | NTU | June 29 |
| Taichung 189 | TC 189 | NTU | June 30 |
| Taichung 189 | TC 189 | TDAIS | Nov. 10 |
| Chianung 242 | CN 242 | NTU | June 29 |
| Chianan 8 | CN 8 | NTU | June 29 |
| Tainan 5 | TN 5 | NTU | July 2 |
| Tainan 9 | TN 9 | NTU | June 30 |
| Kaoshiung 139 | KS 139 | NTU | June 29 |
| Kaoshiung 139 | KS 139 | TDAIS | Nov. 10 |
| Kaoshiung 141 | KS 141 | NTU | June 30 |
| Taitung 27 | TT 27 | NTU | June 29 |
| Taitung 27 | TT 27 | TDAIS | Nov. 10 |
| Taitung 29 | TT 29 | NTU | June 29 |
| Tainung 67 | TL 67 | NTU | July 2 |
| Tainung 67 | TL 67 | AS | June 25 |
| Tainung 70 | TL 70 | NTU | June 29 |
| Tainung 62 | TL 62 | AS | June 25 |
| Hsinchu 10 | SZ 10 | AS | June 25 |
| Indica | | | |
| Tainung Sen 20 | TLS 20 | NTU | June 29 |
| Taichung Sen 10 | TCS 10 | TDAIS | Nov. 10 |

[a]NTU = experimental field, National Taiwan University, Taipei; AS = experimental field, Academia Sinica, Taipei; TDAIS = Taichung District Agricultural Improvement Station, Taichung.

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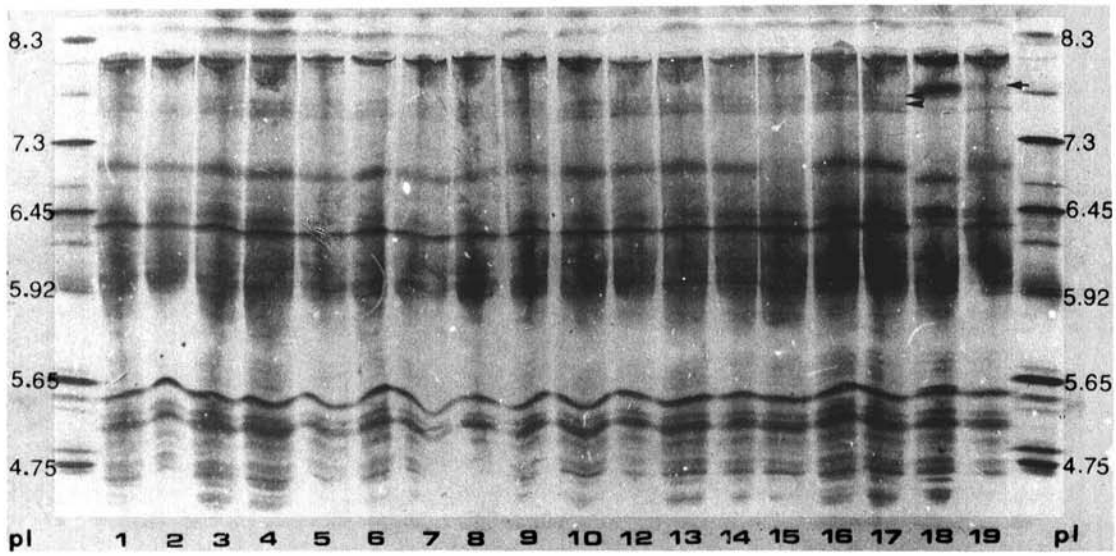


**Fig. 1.** Band patterns of pollen proteins of different cultivars of japonica (**1–10, 12–17**) and indica (**18, 19**) rice analyzed by isoelectric focusing in the 4.75–8.3 isoelectric point (pI) range. The cultivars are as follows (abbreviations listed in Table I): **1,** SZ 10; **2,** TL 62; **3,** TL 70; **4,** TL 67; **5,** TT 29; **6,** TT 27; **7,** KS 141; **8,** KS 139; **9,** TN 9; **10,** TN 5; **12,** CN 8; **13,** CN 242; **14,** TC 189; **15,** TC 65; **16,** SZ 64; **17,** TP 309; **18,** TCS 10; **19,** TLS 20. Pointers = specific bands of japonica rice, arrow = specific bands of indica rice.

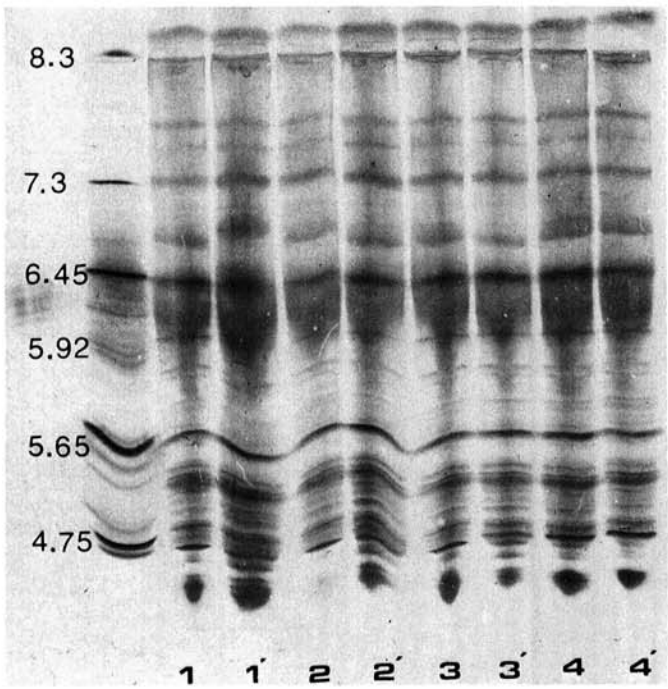


**Fig. 2.** Band patterns of isoelectric focusing electrophoregrams of pollen proteins extracted from four japonica cultivars from various growing locations (in parentheses). **1,** TL 67 (NTU); **1′,** TL 67 (AS); **2,** KS 139 (NTU); **2′,** KS 139 (TDAIS); **3,** TT 27 (NTU); **3′,** TT 27 (TDAIS); **4,** TC 189 (NTU); **4′,** TC 189 (TDAIS). Abbreviations for cultivars and locations are listed in Table I.

### Extraction of Pollen Proteins

Pollen grains (10 mg) were suspended in 100 $\mu$l of 20 m*M* Tris-HCl buffer (pH 8.2) containing 1% mercaptoethanol at 4°C for 1 hr. Soluble proteins were separated from insoluble particulates by centrifugation at 15,000 × *g* for 15 min at 4°C. The supernatant was then ready for further analysis.

### IEF Analysis

Protein extract (10 $\mu$l) was applied to Ampholine polyacrylamide gels made by LKB (Bromma, Sweden) (240 × 100 × 1.0 mm) at 2 cm from the cathode edge. The gel plates (5% T, 3% C) contained 3% w/v carrier ampholytes at pH 3.5–9.5. The separation of protein was performed with an IEF apparatus (LKB 2117 Multiphor II, Sweden) at 4°C using 1,500 V, 50 mA, and 25 W for 1.5 hr. Immediately after focusing, protein bands were visualized by the silver staining method (Gay et al 1986). The reagents used were of analytical grade from Merck-Fine Chemicals, West Germany.

### HPLC Analysis

The separation of proteins was performed on a Waters HPLC system (Millipore Co., Bedford, MA) using a Merck RP-18 column (250 × 10 mm) operated at 1.0 ml/min and 50°C. For elution, 0.10% trifluoroacetate (TFA) in water was used as starting buffer A; 0.08% TFA in 95% acetonitrile and 5% water was used as buffer B. A linear gradient was used for elution, with buffer B running from 0 to 40% within 40 min, then from 40 to 100% within 10 min, followed by 100% B for an additional 10 min. The eluted proteins were detected by their absorbance at 214 nm with a sensitivity of 0.01 AUFS. In this case, both the proteins and peptides were detected.

To determine the homology of the cultivars studied, the protein and peptide peaks on HPLC were compared. Regardless of the height of a peak (that is, the quantity of proteins or peptides), the similarity in peak patterns of different strains was calculated by measuring the degree of match. The simple similarity coefficient ($S = m/(m + u)$) was used, where $m$ is the number of matched peaks and $u$ the number of mismatched peaks. A dendrogram was then obtained, in which similarity coefficients were used for cluster analysis. The values of Euclidean distance, $d = (1 - S)$, were used for this analysis (Sneath and Sokal 1973).

## RESULTS AND DISCUSSION

The pollen proteins extracted by Tris-HCl buffer are mainly from the pollen wall (Knox and Heslop-Harrison 1970). These proteins are readily desorbed from the pollen wall during extraction in aqueous buffer solution.

Both japonica and indica rice had quite different protein components in the pollen extract. They differed in their IEF band patterns, especially in isoelectric point (pI) between 7.3 and 8.3 (Fig. 1).

The variation in band pattern of four cultivars of japonica rice collected from different growing locations was also studied. The results clearly showed no significant difference among them (Fig. 2). This indicates that location of plant growth has no effect on pollen proteins. Cargnello et al (1988) also found that the expression of clone-specific characteristics of pollen proteins in

TABLE II
Composition of Peak Components Detected at 214 nm by High-Performance Liquid Chromatography Analysis of Pollen Proteins of Different Cultivars of Japonica Rice[a]

| Cultivar[b] | Peaks | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 |
| TC 65 | −[c] | + | + | + | − | + | + | + | − | + | + | + | + | + | − | − | + | + | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| TN 9 | − | + | + | + | − | + | − | + | − | + | + | + | + | + | + | − | + | + | − | + | + | + | + | + | + | − | + | + | + | + | + | + | + | + | + |
| SZ 64 | − | + | + | + | + | + | + | + | − | + | + | + | + | + | + | − | + | + | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| TP 309 | − | + | + | + | − | + | + | + | + | + | + | + | + | + | − | − | + | + | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| TL 62 | + | + | + | + | + | + | + | + | − | − | + | − | + | − | − | + | + | − | − | + | − | + | − | + | − | + | + | + | + | + | + | + | + | + | + |
| TL 67 | + | + | + | + | + | + | + | − | + | + | + | − | − | + | + | − | + | − | − | + | + | + | + | + | − | + | + | + | + | + | + | + | + | + | + |
| TL 70 | + | + | + | + | + | + | + | + | + | + | − | + | − | + | − | + | − | − | + | + | + | + | + | + | − | + | + | + | + | + | + | + | + | + | + |

[a] Experimental data described in text.
[b] Abbreviations listed in Table I.
[c] +, − = presence, absence of peak, respectively.

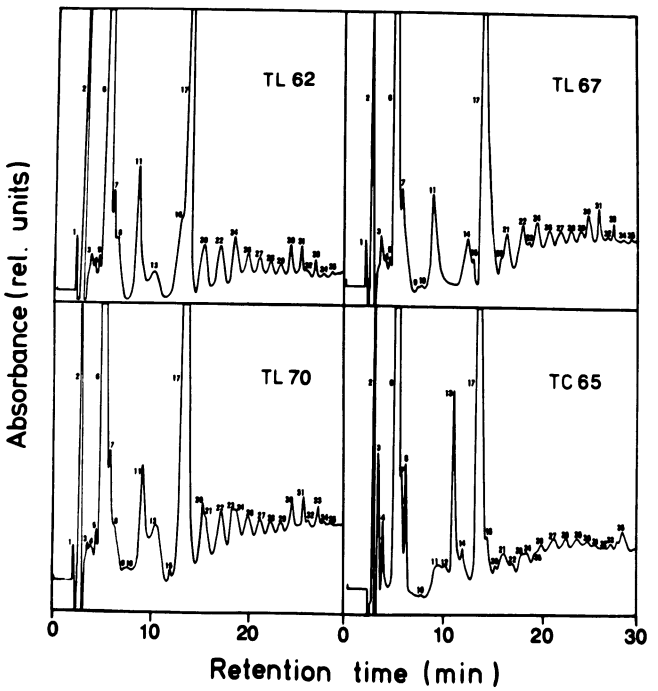


**Fig. 3.** Representative high-performance liquid chromatograms of pollen proteins from four cultivars of japonica rice. Experimental data are given in the text. Abbreviations for cultivars are listed in Table I.

TABLE III
Simple Similarity Coefficients in the Pattern of the First 25 Eluted Peaks on High-Performance Liquid Chromatograms of Pollen Proteins Extracted from Different Cultivars of Japonica Rice

| | Cultivars[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | TL 62 | TL 67 | TL 70 | TC 65 | TN 9 | SZ 64 | TP 309 |
| TL 62 | | 0.57 | 0.70 | 0.57 | 0.48 | 0.54 | 0.52 |
| TL 67 | | | 0.85 | 0.61 | 0.61 | 0.67 | 0.65 |
| TL 70 | | | | 0.65 | 0.65 | 0.70 | 0.70 |
| TL 65 | | | | | 0.89 | 0.86 | 0.95 |
| TN 9 | | | | | | 0.90 | 0.86 |
| SZ 64 | | | | | | | 0.83 |

[a] Abbreviations listed in Table I.

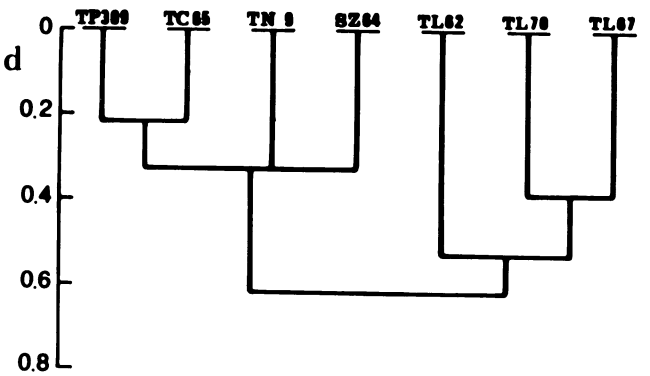


**Fig. 4.** Dendrogram obtained from cluster analysis on the basis of similarity in the first 25 eluted peaks on high-performance liquid chromatogram of pollen proteins from seven cultivars of japonica, showing the distance between them (d = Euclidean distance). The conditions for analysis were the same as described in the text. Abbreviations for cultivars are listed in Table I.

*Vitis vinifera* was rather stable and was independent of change in environmental factors. The independence of the sampling locality makes pollen proteins a useful characteristic for the systematic study of plants.

Between the two cultivars of indica rice (bands 18 and 19 in Fig. 1), differences in IEF band patterns existed, particularly between 6.45 and 7.3 pI (Fig. 1). The IEF analysis, however, did not satisfactorily distinguish different cultivars of japonica rice. The pollen proteins of these plants had quite similar IEF band patterns in all pI ranges analyzed.

HPLC seems a more powerful analytical method than IEF for separating proteins as well as peptides. Because of its higher resolution power, the HPLC method has been widely used in biochemical analysis. This method of analyzing pollen extracts showed at least 35 peak components for each cultivar (Fig. 3). The chromatograms obtained by this analysis showed that each cultivar of japonica rice had its own characteristic peak pattern. These cultivars differed from each other qualitatively as well as quantitatively.

The composition of peak components listed in Table II shows that the qualitative difference among the cultivars was particularly pronounced in the first 25 peaks, according to elution order. Cultivar TL 70 is a progeny of TL 67 and Chianung strain 662028, which was crossed with TL 62. The peak pattern of TL 70 was more similar to that of TL 67 than to that of TL 62 (Table III), which coincides with the phylogenetic relationship between them. Cultivars TP 309, TN 9, and SZ 64 are progeny plants derived from different interline hybridization, with cultivar TC 65 as a common ancestor. The comparison of peak pattern on chromatograms also showed that they were quite similar and should be closely related. The similarity coefficients calculated on the basis of the first 25 peaks were all higher than 0.80 for these plants (Table III).

A cluster analysis was conducted based on the extent of similarity in the patterns of the first 25 peaks on chromatograms of pollen proteins. A dendrogram showing the relationships among the seven cultivars of japonica rice studied was obtained after this analysis was completed (Fig. 4). Based on the values of Euclidean distance, these cultivars can be differentiated into two distinct groups. The short distance between TC 65 and its descendants, TP 309, TN 9, and SZ 64, indicates that they were closely related in genetic identity. In relation to these four cultivars, the other three (TL 62, TL 67, and TL 70) belong to a separate

group. The results of cluster analysis also coincide with the already known phylogenetic relationships among these plants. The qualitative comparison of peak patterns on chromatograms shows that cultivars with a common ancestor tend to have similar peak patterns. A lower similarity coefficient was obtained for cultivars that were not genetically closely related.

The results of the present study show that the extent of similarity in peak patterns among cultivars matches well with their genetic relationships as noted by Huang (1987). Therefore, the peak pattern obtained by HPLC analysis is a fingerprint of a given cultivar. This fingerprint may serve as a marker of the genotype of a cultivar. The results also demonstrate that it is possible to use the pollen proteins from growing plants for cultivar differentiation and even for the phylogenetic study of rice cultivars.

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