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Identification of specific proteins from seed embryos by two-dimensional gel electrophoresis for the discrimination between *indica* and *japonica* rice

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Summary. Proteins extracted from seed embryos of 29 different cultivated rice (*Oryza sativa* L.) and one wild rice (*O. rufipogon* Griff.) were compared by two-dimensional gel electrophoresis analysis. Among more than 300 protein spots on the gel we found some interesting variations in ten spots which were individually designated as proteins A–J. Protein E was observed in all *indica* cultivars but was not found in those of the subspecies *japonica*. In contrast, protein F was only detected in *japonica* cultivars. Protein A existed in all *japonica* cultivars but, with the exception of IR-36, could not be found in other *indica* cultivars. Therefore, proteins A, E and F can be used as markers for the identification of *indica* and *japonica*. Some so-called "Javanica" cultivars showed the characteristics of *japonica* subspecies with regard to proteins A and F, while one other cultivar of Javanica expressed a type intermediate between *indica* and *japonica* interms of proteins A and E. One feature discriminating between Javanica and *japonica* cultivars was found in the D, G, and J proteins which were expressed strongly in Javanica cultivars but were scarcely expressed in those of *japonica*. Expression of subspecies-specific proteins E and F in F_1 hybrids was also investigated.

Key words: Rice – *Oryza sativa* L. – Seed embryo proteins – Rice subspecies – Two-dimensional gel electrophoresis

Introduction

Cultivated rice (*O. sativa* L.) consists of two subspecies, *japonica* and *indica*. The classification of these subspecies has been based mainly on morphological and physiological characteristics, geographic adaptation, the degree of F_1 sterility, and certain other features (Takahashi 1984). However, there is no single or straightforward criterion for distinguishing subspecies of the *indica* and *japonica* types (Morishima and Oka 1981). As biochemical tools, isozymes have been used for the classification of rice varieties (Glaszmann 1987; Second 1991). In addition, restriction fragment length polymorphisms (RFLPs) are of increasing importance for studies of the phylogenetic relationship among rice varieties (Ishii et al. 1986; Chowdhury et al. 1988; Dallas 1988; Second 1991).

Subspecies-specific proteins, if they exist, should be helpful for studies of genetical, taxonomical and evolutionary relationships among rice varieties. Two-dimensional gel electrophoresis, combined with very sensitive silver staining, facilitates the analysis of a great number of protein spots on a single gel. Comparisons of proteins by two-dimensional gel electrophoresis have been used for taxonomic and genetic studies in higher plants (Ladizinsky and Hymowitz 1979; Bauw et al. 1987; Ramagopal 1990). However, only limited information is available concerning the proteins of rice.

Rice varieties show striking differences in sensitivity toward low temperature during the growth stages. In a survey of the proteins of seed embryos with differences in cold tolerance at the stage of germination, we found qualitative and quantitative variations in some protein spots. These differences, however, could not be attributed to differences in cold sensitivity.

In this paper we describe the identification of proteins with a specificity for the subspecies *indica* and *japonica*, respectively.

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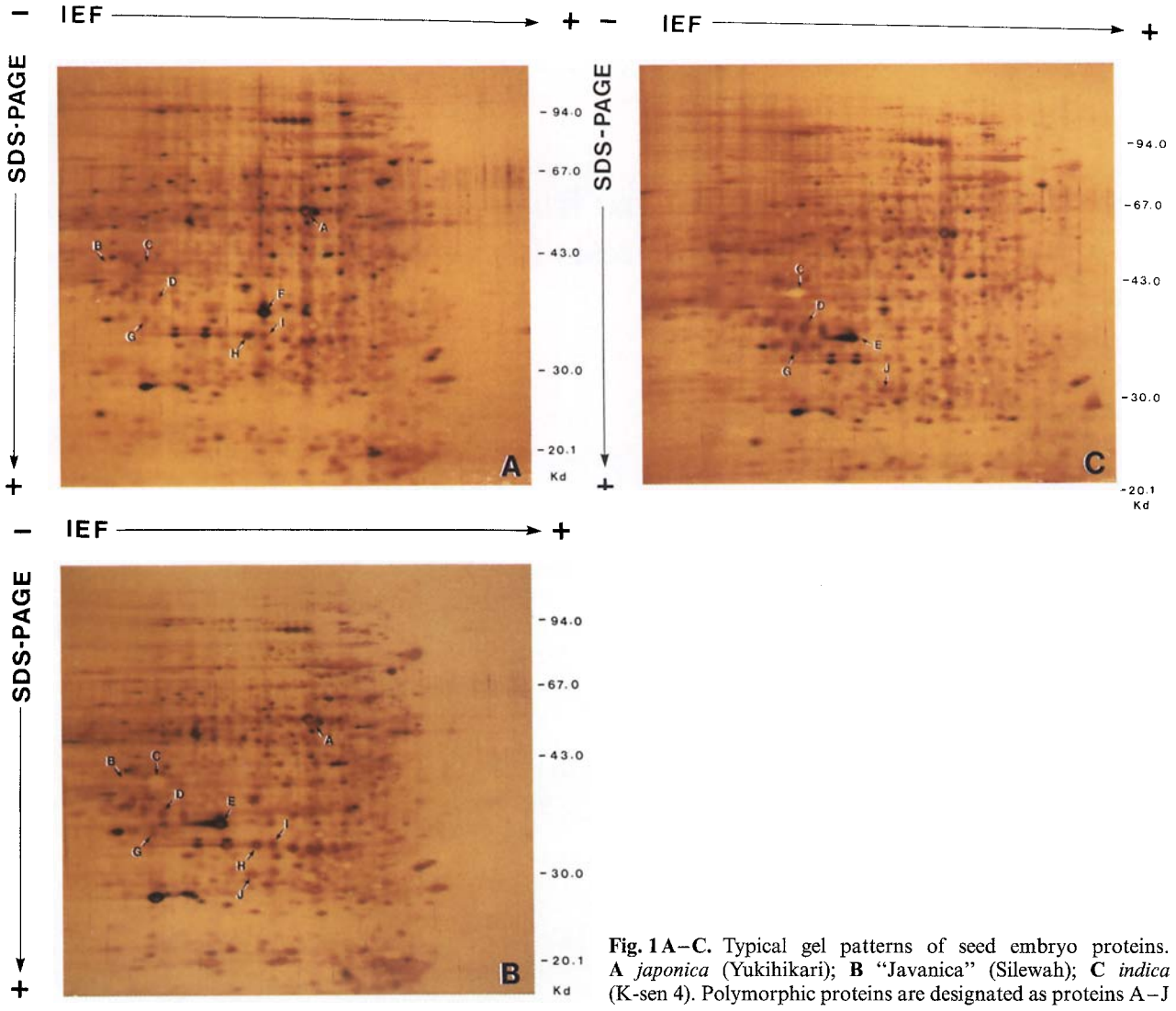

Fig. 1 A–C. Typical gel patterns of seed embryo proteins. **A** *japonica* (Yukihikari); **B** "Javanica" (Silewah); **C** *indica* (K-sen 4). Polymorphic proteins are designated as proteins A–J

Materials and methods

Plant materials

Nineteen *japonica* cultivars of rice (*Oryza sativa* L.) including three cultivars of the so-called "Javanica" type, which is regarded as a tropical subgroup of the Japonica type or ecotypes of *japonica* (see Oka 1988), and ten *indica* cultivars were used in this study. One accession of wild rice (W1944), *Oryza rufipogon* Griff., was also investigated.

Isolation of proteins from seed embryos

Embryos prepared from five to ten seeds of each accession were homogenized by a glass homogenizer for 5 min at 4 °C in 0.1 ml cold 50 mM potassium phosphate buffer, pH 7.0, containing 1% Triton X-100. The homogenate was twice centrifuged for 30 min at 14,000 rpm, and the supernatant was stored at −80 °C until use.

Two-dimensional gel electrophoresis

Soluble proteins of embryos were examined by two-dimensional gel electrophoresis according to the protocol of O'Farrell (1975). The isoelectric-focusing (IEF) gel solution contained 48.6 g urea, 11.8 ml acrylamide/bis solution [29.2% (w/v) acrylamide, 0.8% (w/v) N′-N′-bis-methylene-acrylamide], 20.3 ml 10% (v/v) Triton X-100, 4.5 ml Bio-Lyte 5/7 (Bio-Rad), 0.5 ml Bio-Lyte 3/10 (Bio-Rad) and 28.8 ml distilled water. For preparation of the IEF gel, 1.0 ml IEF gel solution was degassed for 15 min, and 1 µl of N,N,N′,N′-tetramethylethylenediamine (TEMED) and 1.3 µl of freshly prepared ammonium persulfate were added. Then, the gel solution was loaded into the gel tube (125 × 1.5 mm).

Solid urea was added to the protein sample (100 to 150 µg protein in about 30 µl) until saturated. To a 5 µl sample was added 1 µl IEF solution containing 0.1 ml 10% (w/v) SDS, 0.02 ml Bio-Lyte 3/10 (Bio-Rad), 0.18 ml Bio-Lyte 5/7 (Bio-Rad), 0.1 ml 2-mercaptoethanol, and 0.2 ml Triton X-100. The upper and lower reservoirs were filled with 0.1 N NaOH and 0.06% phosphoric acid, respectively. After the sample solution was loaded, the gels were run at 400 V and 800 V for 16 h and 2 h, respectively, at 9 °C. Then the gels were removed from the gel tubes, and equilibrated in 3 ml of reducing SDS buffer [62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, and 0.0125% (w/v) bromophenol blue (BPB)] by shaking for 30 min at room temperature.

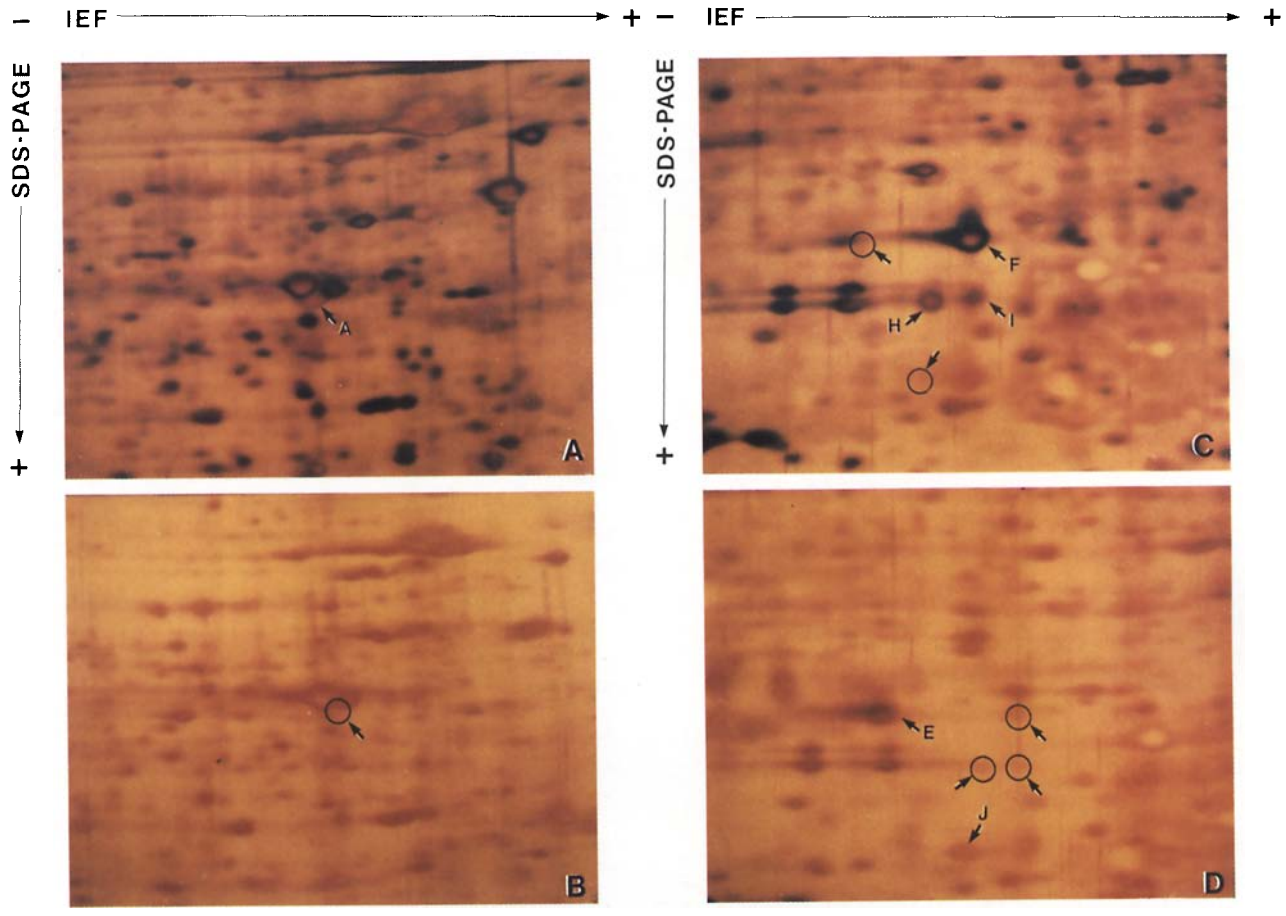

Fig. 2 A–D. Example of polymorphic seed embryo proteins in *japonica* (Yukihikari) and *indica* (K-sen 4). Protein spots of *A*, *E*, *F*, *H*, *I* and *J* between Yukihikari (**A**, **C**) and K-sen 4 (**B**, **D**) are compared and the absence of a protein spot is indicated by a *circle*

The second dimension of gel electrophoresis (150 × 160 × 1.5 mm) was carried out using a separation gel of 11% acrylamide and a stacking gel of 4% acrylamide according to the procedure of Laemmli (1970). The gels were run with a constant current at 15 mA/gel and 4 °C until the BPB reached the bottom of the gel. The gel was stained by the color silver-stain kit (Gelcode, Pierce Chemical Company, USA) with a slight modification.

Protein concentration

Protein concentration was determined according to Bradford (1976) using the Bio-Rad protein assay kit. Bovine plasma immunoglobulin was used as a standard.

Results and discussion

Identification of subspecies-specific proteins

During the comparison of rice seed embryo proteins by two-dimensional gel electrophoresis analysis, we found ten protein spots (denoted as proteins A to J in Figs. 1 A, B, C) with variation in different cultivars. The gels stained by silver enabled us to detect more than 300 spots. Figures 1 A, B, and C demonstrate the typical protein spot patterns of *japonica* (Yukihikari), so-called "Javanica" (Silewah) and *indica* (K-sen 4), respectively. Figures 1 and 2 reveal quantitative and qualitative variations of the ten protein spots among cultivars. This protein polymorphism is summarized in Table 1. Proteins E and F are the most abundant and are stained dark red (Figs. 1, 2 C, D) Interestingly, all typical *japonica* cultivars (Nipponbare, Sasanishiki, Taichung 65, Yukihikari, Kitahikari, Tomoyutaka, etc.) contained protein F (Fig. 2 C and Table 1). In contrast, *indica* cultivars (IR-36, IRAT 127, Leng Kwang, Ma Che, Gaiya D. T., K-sen 4, etc.) contained protein E almost exclusively (Fig. 2 D and Table 1). The only exception is Silewah which belongs to the so-called "Javanica" group and contained protein E instead of protein F (Fig. 1 B and Table 1). Protein A was found in all typical *japonica* cultivars but, with the exception of IR-36, not in those of *indica* (Fig. 2 A, B and Table 1). The parentage of IR-36 reported by the International Rice Research Institute (Khush and Gomez 1985) indicated that it has an ancestral par-

Table 1. Comparison of the protein spots between *japonica* and *indica* cultivars

Sub-species	Cultivars	Protein spots[a]									
		A	B	C	D	E	F	G	H	I	J
japonica	Nipponbare	+	+	+	±	−	+	±	+	+	−
	Sasanishiki	+	+	+	±	−	+	±	+	+	−
	Taichung 65	+	+	+	±	−	+	±	+	+	−
	Yukihikari	+	+	+	±	−	+	±	+	+	−
	Kitahikari	+	+	+	±	−	+	±	+	+	−
	Tomoyutaka	+	+	+	±	−	+	±	+	+	−
	Tomohikari	+	+	+	±	−	+	±	+	+	−
	Tomoemasari	+	+	+	±	−	+	±	+	+	−
	Matumae	+	+	+	±	−	+	±	+	+	−
	Iburiwase	+	+	+	±	−	+	±	+	+	−
	Nekken 1	+	+	+	±	−	+	±	+	+	−
	Akage	+	+	+	±	−	+	±	+	+	−
	Shiokari	+	+	+	±	−	+	±	+	+	−
	Hatushimo	+	+	+	±	−	+	±	+	+	−
	Dohoku 43	+	+	+	±	−	+	±	+	+	−
	Hokuiku Mochi 80	+	+	+	±	−	+	±	+	+	−
(Javanica)	Ketan Nangka 2	+	±	+	+	−	+	+	+	+	+
	Katoentjar 2	+	+	+	+	−	+	+	+	±	+
	Silewah	+	+	+	+	+	−	+	+	+	+
indica	IR-36	+	−	+	+	+	−	+	+	+	+
	IRAT 127	−	−	+	+	+	−	+	+	+	−
	IR 19746-26-2-3-3	−	−	+	+	+	−	+	+	±	−
	IR 9202-25-1-3	−	−	+	+	+	−	+	±	±	±
	NR 10073-181	−	−	+	+	+	−	+	±	−	−
	Leng Kwang	−	−	+	+	+	−	+	+	−	+
	Ma Che	−	−	+	+	+	−	+	−	−	−
	Chianung Sen Yu 13	−	−	+	+	+	−	+	±	±	+
	Gaiya Dhan Tosar	−	−	+	+	+	−	+	±	−	+
	K-sen 4	−	−	+	+	+	−	+	−	−	+
Wild rice	*O. rufipogon*	+	+	±	−	+	−	−	−	−	−

[a] +, ±, and − indicate that the protein spot was present, present in a small amount, or not present, on two-dimensional gels, respectively

ent of the wild rice *Oryza nivara*. *O. nivara* is an annual type of *O. rufipogon* which contains protein A (Table 1). Thus, protein A in IR-36 seems to be inherited from *O. nivara*. We conclude from these results that proteins A, E and F can be used as markers for the classification of *japonica* or *indica* subspecies in rice varieties. In addition, proteins E and F show almost the same molecular mass, 33,000 Da, but with different pI points, namely 6.3 (protein E) and 6.1 (protein F). Since protein E exists in the wild rice *O. rufipogon* (Table 1), protein E might be the ancestral type, while protein F may be a derived product generated by an exchange of amino-acid residues in protein E.

It has been suggested that the so-called "Javanica" types represent a tropical subgroup or ecogroup of *japonica* (see Oka 1988, for review). According to our protein analysis, Ketan Nangka 2 and Katoentjar 2, both regarded as Javanica, show the characteristics of *japonica* subspecies because they contain proteins A and F (Table 1). On the other hand, as stated above, Silewah, which is also classified as a Javanica, expresses both *japonica* type protein A and *indica* type protein E (Fig. 1 B and Table 1). The F_1 hybrids of Silewah and Iburiwase (*japonica*) or K-sen 4 (*indica*) showed more than 70% fertility. Thus, Silewah seems to be an intermediate type between *japonica* and *indica*. On the other hand, distinct differences between so-called "Javanica" and *japonica* are observed concerning proteins D, G and J (Table 1). Proteins D and G were found in small amounts in typical *japonica* cultivars but in large amounts in *indica* and Javanica cultivars such as Ketan Nangka 2, Katoentjar 2 and Silewah. In contrast to Javanica protein J is lacking in typical *japonica* (Table 1). Thus, the presence or absence of these proteins in seed embryos can be used to distinguish so-called "Javanica" from *japonica*.

Table 2. Expression of subspecies-specific proteins E and F in F_1 hybrids and parents

Parents or F_1		Subspecies-specific proteins[a]	
Subspecies	Cultivars	E (*indica*)	F (*japonica*)
japonica	Iburiwase	−	+
	I. livorno	−	+
	USSR-22	−	+
(Javanica)	Katoentjar 2	−	+
indica	K-sen 4	+	−
	Leng Kwang	+	−
(wild rice)	*O. rufipogon*	+	−
japonica/*japonica*	Iburiwase/USSR-22	−	+
	I. livorno/USSR-22	−	+
japonica/Javanica	Iburiwase/katoentjar 2	−	+
japonica/indica	Iburiwase/K-sen 4	+	+
japonica/wild rice	Iburiwase/*O. rufipogon*	+	+
	I. livorno/*O. rufipogon*	+	+
indica/*indica*	K-sen 4/Leng Kwang	+	−
indica/*japonica*	K-sen 4/Iburiwase	+	+
	K-sen 4/I. livorno	+	+
	K-sen 4/USSR-22	+	+
indica/wild rice	K-sen 4/*O. rufipogon*	+	−

[a] + and − indicate presence and absence of the protein spot in the two-dimensional gel, respectively

Analysis of the expression of subspecies-specific proteins E and F in F_1 hybrids

As mentioned above, proteins E and F are markers for *indica* and *japonica*, respectively. Therefore, the expression of these proteins in hybrid seeds was analyzed (Table 2). One example of a two-dimensional gel pattern of seed

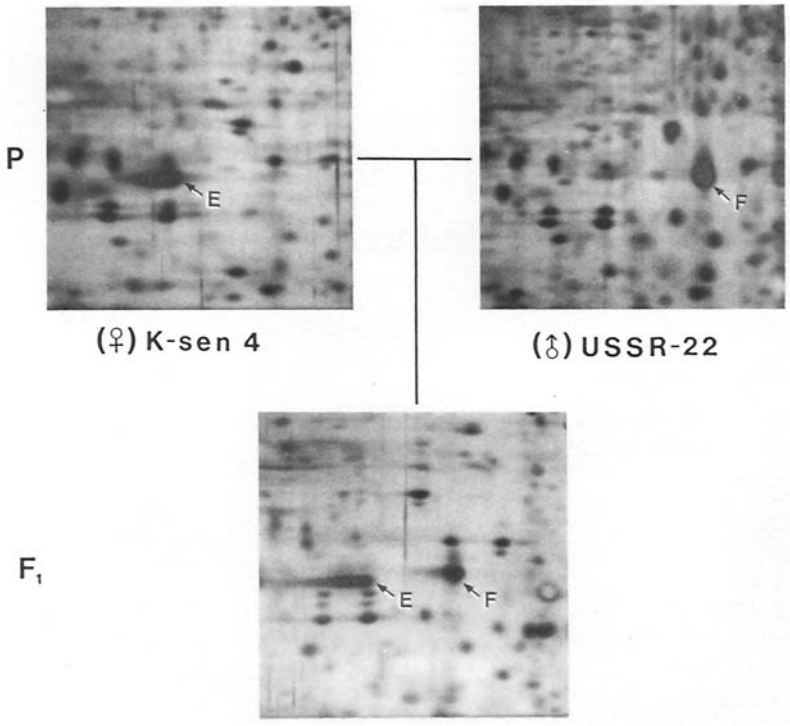

Fig. 3. Expression of parental (*P*) proteins E and F in an F_1 hybrid embryo

embryo proteins of an F_1 between *indica* (K-sen 4) and *japonica* (USSR-22) is shown in Fig. 3. The results are summarized in Table 2. It is clear that both *indica* protein E and *japonica* protein F are expressed in the F_1 embryos of *japonica*/*indica*, *japonica*/wild rice and *indica*/*japonica* hybrids. It appears, therefore, that proteins E and F are governed by codominant alleles at the same locus.

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