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Protein polymorphism in sugarcane revealed by two-dimensional gel analysis

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Summary. In order to identify molecular markers for the analysis of the sugarcane genome, proteins extracted from apical segments of shoot tissues were resolved by a combination of equilibrium (IEF) and nonequilibrium (NEPHGE) two-dimensional polyacrylamide gel electrophoresis. A number of taxa of the "*Saccharum* complex" group (*Saccharum* species and the related genera of Andropogoneae) with presumed contributions to the sugarcane genome were surveyed. Protein profiles were compared to a reference map consisting of 1,482 protein spots from the noble cane, *Saccharum officinarum* L. Fifty-three polypeptides, representing about 3.6% of the total resolved spots, showed interspecific variation, whereas 78 polypeptides, about 5.3% of the total, showed intergeneric variation. Of the total polymorphic protein spots, qualitative (presence/absence) variation was more prevalent among the wild than among the cultivated species of the genus *Saccharum*, but the quantitative (spot intensity) variation was similar for both groups. The population of protein spots showing qualitative and quantitative variations was similar among the related genera of Andropogoneae. These polymorphic proteins can be used in genetic and evolutionary studies of the sugarcane genome.

Key words: *Saccharum* complex – Protein gene markers – Genome analysis – Gene expression – Genome evolution

Introduction

Today's sugarcane (a hybrid of *Saccharum* spp.) is a complex polyploid of $2n \approx 120$ chromosomes. The origin and function of the sugarcane genome is still poorly understood, but currently its evolution is considered dependent upon interaction of the genomes of several plants included under the so-called "*Saccharum* complex" group (Mukherjee 1957; Daniels et al. 1975). This group includes *Saccharum officinarum* L., *S. sinense* Roxb., *S. barberi* Jeswiet, *S. edule* Hassk., *S. spontaneum* L., and *S. robustum* Brandes and Jeswiet ex Grassl, and the related Andropogoneae genera, *Erianthus, Miscanthus, Narenga,* and *Sclerostachya*. The cultivated canes include *S. officinarum* L. (noble cane), *S. sinense, S. barberi,* and *S. edule,* whereas the remainder of the *Saccharum* species are wild.

Markers to identify and characterize the sugarcane genome have been sought for many decades. These markers fall under three broad categories: morphological, cytogenetic, and biochemical. Morphological markers were based on the traditional botanical descriptions of visible characters and were the first markers to be utilized. They are of limited value because in sugarcane they are not inherited in a simple Mendelian manner (Hogarth 1987). Cytogenetic markers observed from mitotic and meiotic chromosomes provided additional information, but the small and variable size of sugarcane chromosomes and their abnormal pairing behavior make cytogenetic observations difficult (Sreenivasan et al. 1987). At present, no karyotypic marker that can distinguish, e.g., the chromosomes of *S. officinarum* from those of *S. spontaneum* has been identified. During the 1970s, biochemical markers became a popular tool in plant genetics, and studies utilizing such markers were also initiated in sugarcane. Isozymes (Waldron and Glasziou 1972; Waldron et al. 1974) and secondary metabolites (Williams et al. 1974; Smith and Martin-Smith 1978) of leaves have been examined and were found to be promising as markers. Because isozymes are the direct product of protein genes, they are particularly useful as good genetic markers. However, the number of

Table 1. Details of sucarcane plants of the "*Saccharum* complex" group

Nomenclature	Genotype and/or origin	Chromosome (2n)	Mean fresh weight per shoot apex (g)
Saccharum officinarum L	Badila, New Guinea	80	0.32
S. officinarum L.	Yellow Caledonia	80	0.41
S. sinense Roxb.	Gungera, India	–	0.17
S. sinense Roxb.	Hawaiian Uba	118	0.22
S. barberi Jeswiet	Saretha	91–92	0.14
S. barberi Jeswiet	Hemja (Mungo), N. India	81–83	0.26
S. spontaneum L.	SES 205A, W. Bengal	64	0.10
S. spontaneum L.	Sumatra No. 2	105–110	0.06
S. robustum Brandes and Jewsiet ex Grassl	Sepik-River (Red Fleshed)	60	0.31
S. robustum Brandes and Jeswiet ex Grassl	Wau-Bulolo	60	0.31
Erianthus benganlese (Retz.) Bharadw	Bengal	60	0.15
E. arundinaceus (Retz.) Jeswiet	Mol. 563, java	60	0.17
Narenga porphyrocoma (Hance ex Trimen) Bor.	India	30	0.02
Sclerostachya fusca (Roxb.) A. Camus	US62-2-7	30	0.04
Miscanthus × *Saccharum*	Wiesel Lake Highlands, New Guinea	–	0.04

Data were obtained by weighing about 15 apical segments of shoots per sample. The source for other information was Heinz (1987) and the germ plasm file of the Experiment Station of the Hawaiian Sugar Planters' Association

assayable isozymes in plants is limited, and not all protein genes encode for isozymes.

In recent years, high-resolution two-dimensional gel methods have been applied to evaluate the products of numerous protein genes in animals and higher plants (Klose 1982; Leonardi et al. 1987). This method allows the simultaneous visualization of a thousand proteins on a single gel. Because the resolution is accomplished under highly denaturing conditions, each protein is characterized at the single polypeptide level. Further, sensitive protein staining methods permit the detection of enzymatic as well as nonenzymatic proteins. Recent work of Leonardi et al. (1987, 1988) on maize has demonstrated the potential of the two-dimensional method in the genetic analysis of plant proteins. In the present study, a two-dimensional method was used to characterize the taxonomic distribution and polymorphism of the proteins expressed by the sugarcane genome.

Materials and methods

Plant material

Table 1 lists all of the sugarcane plants investigated. *Saccharum edule* was not available for this study. These plants were grown at the Maunawili Breeding Station, Oahu, and were harvested in the fall. Apical shoot segments (vegetative meristems with the two visible youngest nodes beneath them) were dissected, rinsed in water, blotted dry, weighed, and frozen in liquid nitrogen. This tissue was enriched in protein and displayed the maximum array of polypeptides in two-dimensional gels. For each sample, between five and ten shoot segments were pooled together before protein extraction.

Protein extraction and two-dimensional gel electrophoresis

Proteins were extracted and purified by the phenol method (Ramagopal 1987) and resuspended at a ratio of 1 ml of lysis buffer per gram (FW) of tissue extracted. Isoelectric focusing (IEF) two-dimensional sodium dodecyl sulfate and nonequilibrium pH (NEPHGE) two-dimensional sodium dodecyl sulfate gels were run as before (Ramagopal 1987), except than the IEF and NEPHGE gels were focused at 750 V for 16 h and 4 h, respectively (Ramagopal 1989). Six gels were run simultaneously in a BioRad (Richmond/CA) multislab electrophoresis unit and visualized after silver staining. The limit of detection for this silver-staining system is estimated to be about 0.5 ng/spot (Leonardi et al. 1988).

Comparison of protein patterns

Large two-dimensional gels (18 × 16 cm) were employed because they resolved protein spots well, thus permitting easy comparison. At least two to three gels were run for each sample until a consistent protein pattern emerged. Only reproducible protein patterns were compared. Equal amounts of protein were applied when a quantitative comparison between samples was made. In addition, the intensities of surrounding spots were considered before scoring a spot as less or more intense than the standard spot. Different amounts of proteins from each sample were also electrophoresed to detect the presence/absence (qualitative variation) of a protein spot. Altogether, about 80 two-dimensional gels were selected for the final comparison. The original, silver-stained gels, together with the enlarged photographic prints, were analyzed and rechecked on three separate occasions.

Results

Figure 1 illustrates the protein patterns of *S. officinarum* resolved by IEF and NEPHGE two-dimensional gels. A

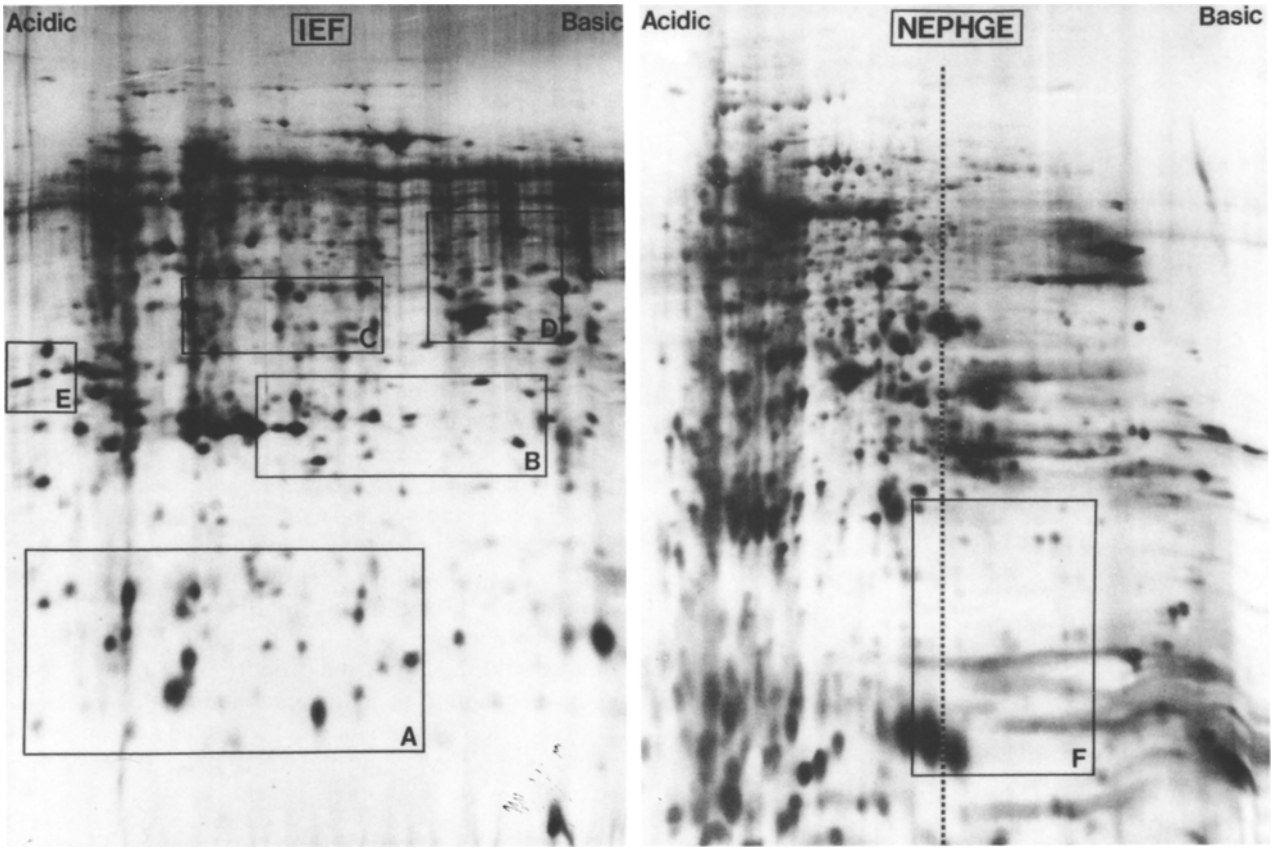

Fig. 1 A–F. Two-dimensional gel patterns of proteins from *S. officinarum* L, genotype Badila. Proteins were resolved by IEF/2D and NEPHGE/2D as described in "Materials and methods". Proteins displayed to the left of the *dotted line* in the NEPHGE panel were resolved only by this gel system. Gel regions showing protein variation are marked within *boxes*

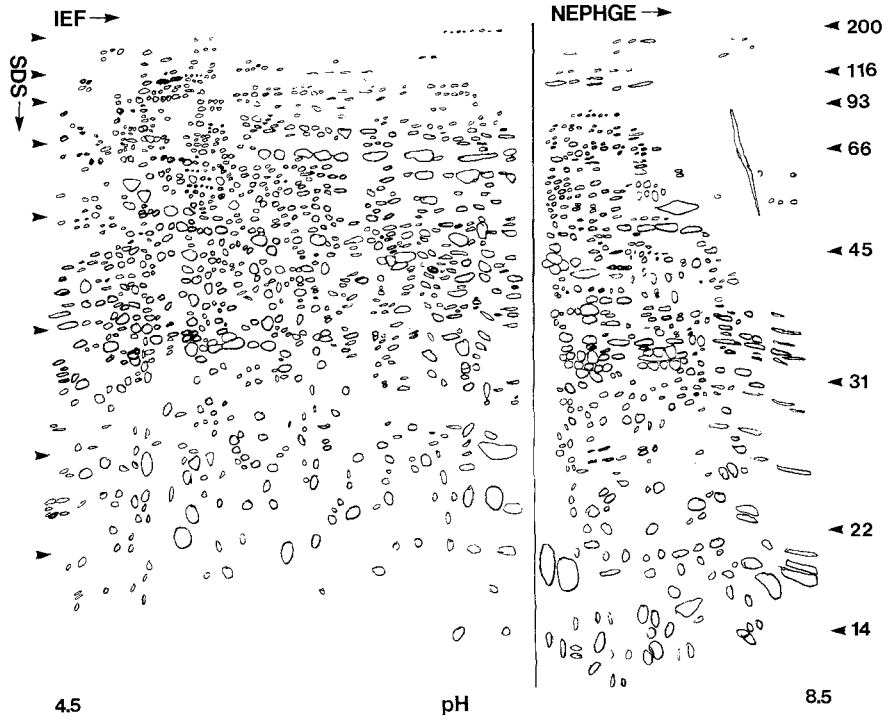

Fig. 2. Diagrammatic representation of a composite map of sugarcane proteins as traced from Fig. 1. Only the basic proteins resolved by the NEPHGE system are included here. Molecular masses are shown in kilodaltons

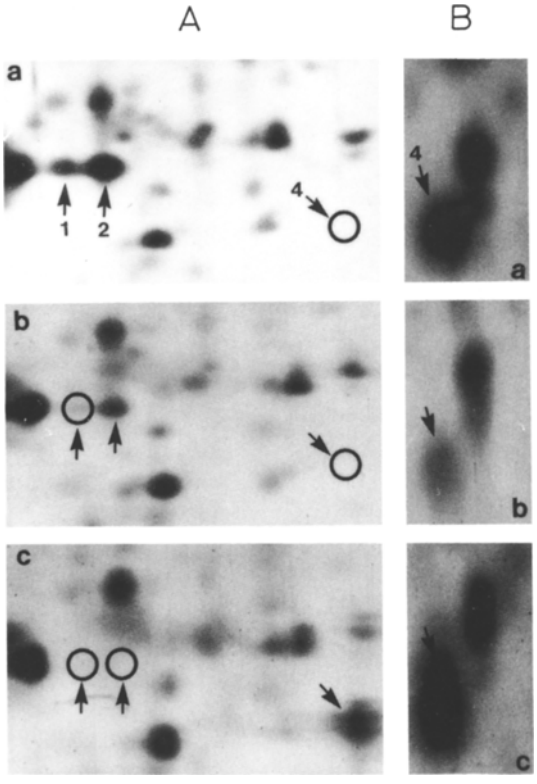

Fig. 3 A and B. Examples of protein polymorphism **A** Qualitative variation is illustrated by the presence or absence of protein spots 1, 2, and 4 of gel region B (see Fig. 4 for nomenclature) *a S. officinarum,* Badila, *b S. spontaneum,* Sumatra, *c S. spontaneum,* SES 205A. **B** Quantitative variation is illustrated by the relative intensity change of protein spot 4 of gel region A. *a S. officinarum,* Badila (+), *b S. barberi,* Hemja (+↓), *c S. barberi,* Saretha (+↑)

composite map of proteins resolved by both gel systems is shown diagrammatically in Fig. 2. A total of 1,482 spots can be visualized. Similar profiles of proteins were observed for the remainder of the sugarcane clones (Table 1). The protein pattern of the *S. officinarum* genotype Badila was arbitrarily chosen here as a reference (standard) sugarcane protein map to which the maps of all other plants of the *Saccharum* complex group were compared.

Two types of polypeptide variation were scored. For qualitative variation, the presence or absence (±) of a protein spot is compared to the standard map (Fig. 3 A). For quantitative variation, the intensity of a protein spot found in both the standard genotype and the sample genotype was compared (Fig. 3 B). Only distinct differences in spot intensity (200% or more) were considered and compiled for final analysis.

The polypeptides analyzed here were distributed in five regions (A–E) of the IEF/2D gel and one region (F) of the NEPHGE/2D gel (Fig. 1). Other regions, particularly the top left of IEF/2D gel, were difficult to evaluate visually and thus were not analyzed. Figure 4 shows the enlarged version of gel regions A–F, with the nomenclature for the various protein spots. Except for the variability noted in Table 2, no electrophoretic differences could be detected.

Table 2 lists the individual protein and polymorphisms from each gel region. A partitioning of all sugarcane proteins according to their variation was done and is summarized in Table 3. Fifty-three proteins (3.6% of the total resolved spots) showed interspecific variation, whereas 78 proteins (5.3% of the total) showed intergeneric variation. Although only two genotypes were analyzed for each species, some proteins exhibited intraspecific variation (Table 2). To compare the overall relative variation among the cultivated and wild species of the genus *Saccharum,* the data for all four genotypes in the *sinense-barberi* group and *spontaneum-robustum* group were combined and averaged. Of the total protein polymorphisms, qualitative variation was more prevalent among the wild (37.3%) than among the cultivated species (26.4%). But the quantitative variation was similar for both groups – 14.2% among cultivated and 13.2% among wild. Likewise, the proportion of protein spots showing qualitative and quantitative variations was similar (23% versus 19%) among the related andropogonaceous genera (excluding the *Miscanthus* × *Saccharum* hybrid).

Discussion

In the present study, about 1,500 polypeptides expressed by the sugarcane genome were resolved by a combination of equilibrium and nonequilibrium gel methods. The number of sugarcane proteins resolved here fall in the maximum range attainable by the current two-dimensional gel techniques and approaches the highest numbers reported in other plant and animal species (Celis and Bravo 1984). Because the size of the haploid genome is unknown, it is difficult to ascertain at this time whether these 1,500 spots represent the full protein coding potential of the sugarcane genome. Since only one type of tissue was analyzed to facilitate the taxonomic comparisons, some additional protein spots were probably missed. However, our preliminary work on sugarcane (unpublished data) and work on organ-dependent protein variability in maize (Leonardi et al. 1988) indicate that such an analysis is not expected to reveal more than 10%–15% additional spots, bringing the total number of polypeptides to about 1,700.

Each spot on the gel presumably represents the product of a distinct polypeptide gene of the sugarcane genome, since denaturing conditions were employed in the preparation and separation of proteins. All soluble proteins of the cell – enzymatic and structural – should be present in the protein profiles obtained from these whole

Table 2. Protein polymorphisms listed according to taxa

Protein	*Saccharum* species										Related Andropogoneae grasses				
	officinarum		*sinense*		*barberi*		*spontaneum*		*robustum*		*E.b.*	*E.a.*	*N.p.*	*Sc.f.*	*M.S.*
	B	YC	G	HU	S	H	205	SUM	SR	WB					
Gel region A															
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	−										−	−	−	+	−
Gel region B															
1	+	+	+	+	+↓	+	−	−	+	+↓	−	−	−	−	+
2	+	+	+	+	+	+	−	+↓	+	+	+	+	−	−	+
3	−	−	−	+	−	−	+	−	−	−	−	−	+	−	+
4	−	−	−	−	−	−	+	−	−	−					
5	−	−	−	−	−	−	−	+	+	+					
6	−	−	+	−	+	+	+	+	−	+	−	+	−	+	−
7	+	+	+	+↑	+	+	+↑	+↑	+	+	+	+	+↑	+↑	+
8	+	+	+	+	+	+	−	−	+	+	+↓	+	−	−	+
9	−	+	−	+	+	+	+	+	+	+					
10	−										−	+	−	−	−
11	+										+	+	+	−	+
12	+										+↑	+↑	+	+	+
13	+										+↑	+↑	−	+	+
14	+										+↓	+	+↑	+↑	+
15	+										+↓	+	+↑	+↑	+
16	−										−	+	−	+	−
17	−										−	−	+	+	−
18	+										+	+	+↑	+↑	+
19	+										+	+	+↑	+↑	+
Gel region C															
1	−	+	+	−	−	+	−	+	+	+					
2	−	+	+	−	−	+	−	+	+	+					
3	−	−	−	−	−	−	−	−	−	+					
4	−	−	−	−	−	−	−	−	−	+					
5	+	+	+↓	+	+	+↓	+↓	+↓	+	+↓	+↓	+↓	+↓	+↑	+
6	+	+↑	+	+↑	+↑	+	−	+	+↑	+↑	−	−	−	−	−
7	+	+↑	+	+↑	−	+	−	−	+↑	+↑	+↑	+↑	−	+↑	+
8	−	−	+	+	+	−	+	+	+	+					
9	−	−	+	+	−	−	+	−	−	−					
10	−	−	−	−	+	+	−	+	−	−					
11	+										+↑	+↑	+	+↑	+

Table 2. (Continued)

Protein	*Saccharum* species										Related Andropogoneae grasses				
	officinarum		*sinense*		*barberi*		*spontaneum*		*robustum*		*E.b.*	*E.a.*	*N.p.*	*Sc.f.*	*M.S.*
	B	YC	G	HU	S	H	205	SUM	SR	WB					
12	+										+↑	+↑	+↑	+↑	+
13	+										+↑	+↑	+	+	+
Gel region D															
1	+	+	+	+	−	+	−	+	+	+	−	+	−	+	−
2	−	−	−	−	−	−	−	−	+	−					
3	−	−	−	−	−	−	−	−	+	−					
4	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−
5	−										−	+	−	−	−
6	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−
7	−										−	−	−	+	−
8	+	+	+↓	+↓	+	+	−	+	+	−	−	+	+	+	+
9	−	−	+	−	+	−	+	−	−	−	−	+	−	−	+
10	−	−	+	+	−	−	+	+	+	+	−	+	−	+	−
11	−	−	+	+	+	+	+	+	−	+	+	+	+	−	+
Gel region E															
1	+	+	+	+	+	+	+	+	+	+	+↑	+↑	+	+	+
2	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
3	+	+	+	+	+	+	+	+	+	+	+↑	+↑	+	+	+
Gel region F															
1	−	−	+	+	−	+	+	+	+	+	−	−	+	−	−
2	+	−	−	−	−	−	−	−	−	−	+	+	+	+	−
3	−	+	+	−	−	+	+↓	+↓	+	+↓					
4	+	+	+	+	+↓	+	+↓	+	+	+	−	−	+	+	+
5	+	+	+	+	+↓	+	+↓	+	+	+					
6	+	−	−	−	−	+↓	−	−	−	−	−	−	+↓	+	−
7	−	−	−	+	+	−	−	−	−	−					

Abbreviations: B–Badila, YC–Yellow Caledonia, G–Gungera, HU–Hawaiian Uba, S–Saretha, H–Hemja, 205–SES 205A, SUM–Sumatra, SR–Sepik River, *E.b.–Erianthus bengalense, E.a.–E. arundinaceus, N.p.–Narenga porphyrocoma, Sc.f–Sclerostachya fusca, M.S.–Miscanthus* × *Saccharum;* Protein nomenclature for the different gel regions is described in Fig. 4. The following symbols are used to describe qualitative and quantitative variations: + = protein present, − = protein undetectable; +↑ = protein present in higher amounts than that in Badila; +↓ = protein present in lower amount than that in Badila

tissue extracts, although some proteins in the subcellular compartments (e.g., nucleus, organelle, membrane) could be underrepresented owing to solubility problems or low abundance. Therefore, the present data allow a preliminary estimation of the overall trend in the variation of protein genes encoded by the sugarcane genome.

Polymorphisms were detected in only 3.6%–5.3% of the total polypeptides despite the existence of vast morphological differences among the taxa. This range should be considered a minimum estimate, because portions of gels with crowded spots and low-abundance spots were excluded from the present visual analysis. A more accurate estimate could be obtained by a modern automatic image analyzer. To my knowledge, there are still no published reports comparing interspecific and intergeneric protein variation in diverse taxa of higher eukaryotes using two-dimensional gels, as was done here. A comparison of two inbred lines of maize showed an 11% variation among the 816 protein spots scored (Leonardi et al. 1987). On the other hand, in mouse a comparison of 830 spots in four inbred strains revealed a 25% variation (Klose 1982).

Each of the polymorphic proteins differed either qualitatively or quantitatively among the taxa. Presence of a spot meant that the gene coding for the protein is present in the genome and, further, that it is expressed in the tissue. The simplest explanation for the absence of a spot would be that the gene is absent in the genome. Indeed, in *Escherichia coli* genetic mutants, lack of a protein spot on two-dimensional gel correlated with the absence of the gene (Neidhardt et al. 1983). In eukaryotes, a gene may not be expressed owing to developmental or environmental constraints. Part of the variation estimated as qualitative here may be due to allelic differences. Allelic variation can also be deduced from two-dimensional protein profiles, but it requires the analysis of defined crosses (Leonardi et al. 1988). Because the taxa investigated were so diverse, we have not attempted to

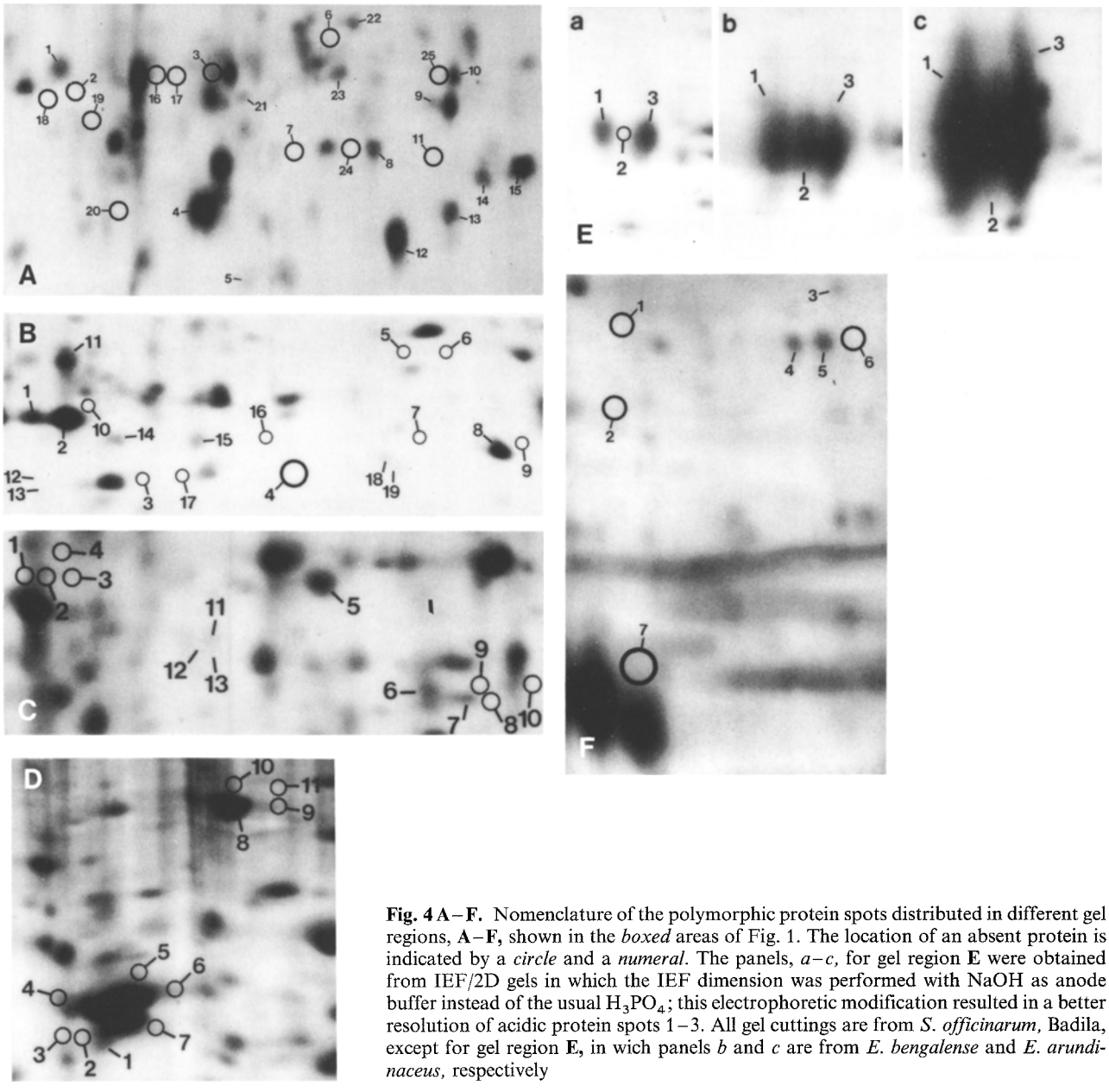

Fig. 4 A–F. Nomenclature of the polymorphic protein spots distributed in different gel regions, **A–F,** shown in the *boxed* areas of Fig. 1. The location of an absent protein is indicated by a *circle* and a *numeral.* The panels, *a–c,* for gel region **E** were obtained from IEF/2D gels in which the IEF dimension was performed with NaOH as anode buffer instead of the usual H_3PO_4; this electrophoretic modification resulted in a better resolution of acidic protein spots 1–3. All gel cuttings are from *S. officinarum,* Badila, except for gel region **E,** in wich panels *b* and *c* are from *E. bengalense* and *E. arundinaceus,* respectively

estimate the allelic variation of sugarcane proteins in the present study. All the spots showing qualitative differences may be considered as the products of structural genes, whereas those varying quantitatively are probably the gene products of regulatory protein loci (Chandlee and Scandalios 1987). The regulatory factors contributing to their variation might be contained in them, as *cis* acting DNA elements, or they could be *trans* factors (proteins), and if the latter are expressed in sufficient quantities, they also may be represented in the two-dimensional pattern.

The findings suggest that the number or the amount of expressed proteins is not correlated with chromosome number. Although the chromosome number in plants studied here ranged from $2n = 30$ to 118, more than 90% of the protein spots did not vary interspecifically or intergenerically. Seed proteins of diploids and polyploids (Ladizinsky and Hymowitz 1979) and seedling proteins of synthetic wheat amphiploids with $2n = 14$ to 42 have also been found to be very similar (Bahrman and Thiellement 1987). Apparently, similar sets of chromosomes from the different plants were encoding those invariable

Table 3. Summary of the number of sugarcane proteins showing polymorphisms

Nomenclature	Total scored	Protein variation		
		Qualitative (A)	Quantitative (B)	A+B
Saccharum species and genotype				
officinarum, Yellow Caledonia	53	7	7	14
sinense, Gungera	53	15	5	20
sinense, Hawaiian Uba	53	15	9	24
barberi, Saretha	53	12	10	22
barberi, Hemja	53	14	6	20
spontaneum, SES 205A	53	24	8	32
spontaneum, Sumatra No. 2	53	21	5	26
robustum, Sepik-River	53	15	8	23
robustum, Wau-Bulolo	53	19	7	26
Related Andropagoneae grasses				
Erianthus bengalense	78	16	16	32
E. arundinaceus	78	22	15	37
Narenega porphyrocoma	78	17	12	29
Sclerostachya fusca	78	17	15	32
Miscanthus × *Saccharum*	78	10	3	13

Data are summarized from gel regions A–F of Table 2. Protein variation is expressed with respect to *S. officinarum*, Badila (Standard, see text)

proteins, and most of the spots probably represent products of "housekeeping" genes involved in such essential biochemical processes as DNA replication and glycolysis.

In conclusion, this initial two-dimensional study has revealed a number of polymorphic proteins among the taxa of the *Saccharum* complex. Further studies of these proteins will be useful in characterizing the evolution of the sugarcane genome at the molecular and genetic levels. In addition, the two-dimensional approach presents a unique opportunity to clone and characterize a large number of authentic protein genes expressed by the sugarcane genome. Because of the high resolving power, each protein can be rapidly purified (Bauw et al. 1987) without resorting to the often cumbersome conventional protein purification procedures; thus, this method facilitates the preparation of specific antibodies and primers essential for screening gene libraries.

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