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GENEVA

WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES AND DNA-PROFILING IN PARTICULAR

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BIOCHEMICAL AND MOLECULAR TECHNIQUES AND DNA-PROFILING:

PRELIMINARY ASSESSMENTS AND COMPARISON OF METHODS AS APPLIED TO

SOYBEANS

Glycine max (L.) Merr.

Document prepared by experts from the United States of America

INTRODUCTION

This text has been constructed with the primary focus on biochemical and molecular techniques as they relate to **soybeans**. When applied to other groups of plants, some techniques may or may not be more reliable or better suited.

In recent years the number of plant breeders incorporating biochemical and molecular techniques (BMT's) into their research has steadily increased. Their uses have ranged from distinguishing and characterizing as many as 96 closely related varieties, to determining the presence or absence of certain genes within a variety. For varieties which appear similar morphologically, and cannot be distinguished easily in field trials, such techniques may be valuable in proving distinctness. For example, when one is able to identify and map the alleles responsible for certain disease resistance, it is increasingly justifiable to base cultivar identification on the presence or absence of such alleles detected by these advanced methods. The majority agree that while the use of BMT's is useful in cultivar *identification*, such work must not be considered a substitute for in vivo *observation* or *field testing* at this time. Proving that a gene for a disease resistance is present may not necessarily guarantee that all subsequent generations of the variety are truly resistant to that disease. Similarly, one must not ignore the fact that genes coding for certain character states have indeed been accurately mapped. In reality, it is unlikely that anyone ever intended for BMT's to become a total substitute for the more traditional kinds of testing. However, some who shy away from biotechnology seem to fear that possibility, regardless of the attitude of the presenter. Targeting attention to the facts, rather than one's fears would be prudent. Ironically, it is often the researcher using BMT's who is the most wary of their limitations, as well as cautious of the consequences of failing to consider genetic drift, mutation frequency, and environmental factors. As the diminution of genetic diversity continues, BMT's will continue to be increasingly useful. If they are shunned, we will suffer the same ill fate as if they are viewed with fanatical reverence.

Table 1. Advantages and disadvantages of simple Sequence Repeat (SSR), Restriction Fragment Length Polymorphism (RFLP), and Amplified Polymorphic DNA analysis.

FEATURE	SSR	RFLP	RAPD
polymorphisms are easy to find - can accommodate multi-allelic states	YES	NO	NO
can distinguish hetero- and homozygous states	YES	YES	NO
restricted to analysis of homologous loci	NO	NO	YES
based on polymerase chain reaction (allows for rapid DNA acquisition)	YES	NO	NO
can reveal homologous loci	YES	YES	YES
requires use of radioactive materials	YES	YES	NO
requires large amounts of intact DNA	NO	YES	NO
automation possible	YES	NO	YES
faster than RFLP analysis (does not require Southern blot technology)	YES	N/A	YES
polymorphisms are difficult to find	NO	YES	NO
effective probes are difficult find	NO	YES	NO
techniques difficult and time consuming	NO	YES	NO
repeatability questionable	NO	NO	YES
number of loci (as of 1994)	21	110	8

ELECTROPHORESIS: ISOZYME ANALYSIS

In soybean research, isozyme analysis has at best received mixed reviews. Soybean experts have reported little single locus or multilocus variability in cultivated varieties, with an even

lower frequency in the most recently released cultivars. Many scientists are reserving isozyme study to analyze biogeographic trends rather than to distinguish individual genotypes or cultivars. However, assays of seed protein peroxidase, seed protein electrophoretic bands (TYPE A: $SP1^a$ and TYPE B: $SP1^b$), and some other characteristics, remain very useful in distinguishing some cultivars.

The following procedures for gel electrophoresis in soybeans were generously provided by the expert from France, Dr. Joël Guiard, GEVES.

At 4°C.
Organ: dry kernel.
Extraction buffer: 0.1 M Tris HCl(pH 7.2), 0.2% β-mercaptoethanol.
1 ml per kernel.

GEL RUNNING CONDITIONS:

At 4°C. Samples migrate towards the anode.

In 12.5% hydrolysed starch gel.

Table XVI: Conditions for electrophoresis of various soybean enzymes

Enzyme	Running buffer	Gel buffer	Voltage	Time
PGM PGD IDH PRX DIA MPI ACP	Histidine-Citrate (0.072 M), pH 6.5	running buffer diluted 1 in 3	constant 18V/cm^2	5 hours bromphenol blue 12 cm

INTERPRETATION OF BANDING PATTERNS:

The genetics underlying each of these enzyme systems has been characterized. Zymograms are thus interpreted in terms of genotype (fig. 15a and b, table XVII).

Table XVII: Structure of the enzymes, and names of genes and alleles in soybean zymograms

Enzymes	Enzymes Structure	Genes	Alleles	
PGM	Monomeric	Pgm1	a,b	
PGD	Dimeric	Pgd1	a,b	
IDH	Dimeric	Idh1	a,b	Intergenic
	Dimeric	Idh2	a,b	interactions
PRX		Ep	Ep, ep (null)	
DIA	Tetrameric	Dia3	Dia3,	Intergenic
			dia3 (null)	interactions
MPI		Mpi	b,c	
ACP	Monomeric	Acp	a,b	

REFERENCES: (for enzyme techniques as supplied by Dr. Guiard)

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RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLP'S)

Restriction fragment length polymorphisms are among the array of DNA markers that may be used to develop DNA profiles. In one study (Keim et al. 1989) 17 RFLP probes were used to characterize a group of 58 soybeans. Seven of the 58 genotypes had identical RFLP patterns and were indistinguishable based on that characteristic. Only two alleles were observed in 15 of the 17 RFLP loci. Three alleles were detected at the remaining two loci. It is this *very limited number of alleles per locus* that in turn severely limits the information yielded by soybean RFLP probes. This relatively low level of informativeness has been documented in studies of humans which incorporate RFLP analysis.

Additionally, it has been reported in TWA/22/17 prov. that probes which had proven successful in the United States did not lead to the same results in France. While soybean research using RFLP's undoubtedly has merit, detailed documentation regarding all methods and conditions is critical. Since many laboratories have a tendency to vary slightly their techniques in accordance with the individual situations and pieces of equipment, such documentation is not only very time consuming, but likely to lack the necessary detail. Ultimately, unless the source of variation which apparently occurs among and within laboratories is discovered and eliminated, the technique cannot be used reliably to measure distinctness, uniformity and/or stability in soybeans. The techniques involved have been described as laborious and highly technical. Whether the variation is due to slightly differing techniques or some uncontrollable factor is somewhat irrelevant. The variation exists, and has not been extricated within the last six years (at least). With that in mind, and the limited information yielded by the technique as described above, it appears that for soybeans, RFLP analysis ***alone*** does not satisfy the standards and criteria established for DUS testing in UPOV.

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD's)

Random amplified polymorphic DNA (RAPD's) analysis involves an assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide

sequence. These polymorphisms are detected as DNA segments which amplify from one parent but not the other. Thus, essentially inherited by Mendelian principles, they may be used to construct genetic maps.
Although RAPD's have the attractive advantage of being less labor intensive and faster to yield results than RFLP's, the problem with repeatability has become commonplace. That, combined with the lack of ability to distinguish hetero- and homozygous conditions severely limits any benefit RAPD analysis ***alone*** has in a soybean analysis which would conform to the necessary UPOV criteria.

SIMPLE SEQUENCE REPEAT (SSR) DNA MARKERS FOR SOYBEAN GENOTYPE IDENTIFICATION

Simple sequence repeats are DNA sequences such as $(AT)_n/(TA)_n$ and $(ATT)_n/(TAA)_n$ that are composed of tandemly repeated 2-5 basepair DNA core sequences. The DNA sequences on either side of the SSR are usually conserved, thus allowing for the selection of a polymerase chain reaction (PCR) primer that will amplify the intervening SSR. It is the variation in the number of tandem repeats, the "n", which results in PCR product length differences. In a study of 96 genotypes (Cregan et al., in press), all proved to be distinguishable based on repeatable differences in SSR allelic profiles. The gene diversity (= heterozygosity), which can be used as a measure of the informativeness of molecular genetic markers, ranged from 0.82 to 0.94 among only four of the markers. Since the submission of those figures, more markers have been elucidated. The ranges listed above are considerably higher than what is typically reported for RFLP markers (in soybeans). Thus far repeatability has not proven to be a problem.

Perhaps one of the most crucial aspects of SSR loci is the significant elimination of ambiguity of allele determination. Due to the fact that SSR alleles are visualized on sequencing gels using a sequencing ladder as a standard, exact allele size (PCR product length) is determined. Allele size can generally be determined within one basepair. In ongoing research, it is hoped that within the next two years, between 250 to 300 new SSR loci can be mapped, with perhaps 5 to 10 highly informative loci that can be used to provide the basis for a soybean DNA profile system. While the two year time span may seem excessive given the urgent need for reliable diagnostic characteristics in soybeans, it should be remembered that of the 96 genotypes analyzed thus far, all were distinguishable. One practical application that has already been performed consisted of identifying two varieties in which the seed labeling had been mixed. There had been no reliable way of determining which seed lot was which. By first sampling each lot to characterize the SSR profile, it was a simple matter to compare the results and accurately identify the seed.

Estimated laboratory set-up costs in U.S. dollars for SSR analysis.

PCR machine		3000-12000
power supply		3000-5000
cassettes		300
sequencing gel apparatus	3000	
total	9300-23000	

This estimate is also similar to the set-up costs of a laboratory for isozyme analysis.

BIOCHEMICAL AND MOLECULAR TECHNIQUES AND ESSENTIALLY DERIVED VARIETIES

Essentially derived varieties (EDV's) represent a controversial topic in their own right, even if we did not have the task of incorporating BMT's into the subject. For those accustomed to being able to determine the identity of various soybean varieties on the basis of the unassisted observation of the phenotype, it can be a very uncomfortable task indeed to choose among BMT's, where the phenotype may only be of conversational interest. Even if it were possible to make the same periodic progressive observations of a laboratory that we can a field plot, few would really know if each sample had been treated identically, and whether or not variation due to human error had been kept to a minimum. TWA/22/17 included similar concerns, and such concerns must be expected. One must, however, bear in mind the wide and erratic variation that may be present in some traditional phenotypic characteristics. Plant height, leaflet size and shape, and in some cases pubescence color, can be influenced by environmental factors. One must also keep in mind the very nebulous concept (**not to mention definition**) of essentially derived. One definition having been considered for soybeans is: "**...A variety would not be considered essentially derived if it contained less than 75% of one parent's genome for that portion of the genome not common among all stated parents. A variety would be considered to be essentially derived if it contains 90% or more of one parent's genome for that portion of the genome not common among all stated parents. If the variety contains equal to or greater than 75% and less than 90% of one parent's genome for that portion of the genome not common among all stated parents, then further steps need to be taken to resolve essential derivation.**" Further steps indeed. Such thoughts make DUS testing seem rather simple. What has rarely been mentioned in discussions of essential derivation or DUS testing, is the mutation frequency and the

role it plays. In an autogamous crop such as soybean, the influence may be quite substantial. It can only be determined by repeated DNA profiling over time. While there has been tremendous progress in mapping the soybean genome, one must bear in mind that the linkage groups alone have still not been mapped.

CONCERNS AND BMT'S

1. Breeder testing: This is a relative term. It should first be pointed out that the U.S. certainly does not assume to characterize the way each member state should or should not allow breeder testing. The trend in the U.S. however, seems to be in the opposite direction of that assumed as reported in TWA/22/17. An increasing number of breeders are sending seed samples to private independent facilities, as well as universities and university departments partially funded by the U.S. Department of Agriculture. Many which do their own testing also send duplicate samples to such facilities for independent multiple comparisons.

2. For the sake of making a difference: This possibility has existed for a very long time. If a breeder wishes to incorporate an insignificant trait while maintaining the essential traits of a competitor's variety, it can be easily done. Flower color, disease resistance, and other characteristics can be bred into a variety quite easily - sometimes more easily than a different DNA profile.

3. Countries going their own way: Undoubtedly, that statement will result in lengthy discussion. Research to elucidate the genetic identity of crops has been increasing and will certainly continue. It is not realistic to believe that researchers will restrict their knowledge of their new varieties because not everyone agrees with the method of study. Many times methods will be wrong; when they are they will be corrected and the research will continue.

4. The cost: While that is especially difficult, there are ways to help alleviate the problem. It is generally much less expensive to have universities/government labs analyze samples at a reasonable profit than for a breeder to set up a complex laboratory. This is especially true when one considers that in several years more advanced techniques and equipment will undoubtedly emerge.

CONCLUSION

The research in biochemical and molecular techniques will continue. Combined with traditional research and techniques in plant breeding, the possibilities are significant. As more varieties of soybeans are produced, the genetic similarity will increase.

Distinguishing between them has become more difficult and it will continue to do so. I find it impossible to comprehend any future progress in soybean breeding if restrictions are placed too severely, either on the use of BMT's or traditional characteristics.

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