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STANDARDISATION OF MOLECULAR MARKER SYSTEMS FOR VARIETY TESTING

Document prepared by experts from the Netherlands, the United Kingdom and France

Standardisation of molecular marker systems for variety testing

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Introduction

Recent developments in molecular biology have resulted in novel techniques of DNA profiling that can be used for the characterisation of plant material. Molecular markers have many advantages over the more traditionally used morphological and biochemical markers for plant variety identification because of their independence from environmental influences and their almost unlimited availability. Molecular techniques are also likely to be extremely discriminating and much more rapid, with results available in a few days. By comparison, when using morphological or physiological characteristics, plants have to be grown to full maturity, which can take 3-4 months or longer. Alternative methods for laboratory based identification, such as isozyme analysis, are inadequate in many crops. There are several approaches to DNA profiling. The Sequence Tagged Microsatellite Site (STMS) approach yields highly informative and discriminating markers, is suitable for automation and the results obtained can easily be stored in an electronic database, which facilitates comparison of results and collaboration between laboratories. Thus this approach offers a new, rapid, powerful and cost-effective means of variety identification that could be used for quality control purposes, as well as for other important applications in plant variety and seeds work.

European Union funded demonstration project

Recently, the European Union has agreed to fund a biotechnology demonstration project entitled 'Molecular markers for variety testing'. This multi-national project aims to demonstrate to potential users, the many advantages of molecular techniques for variety testing. The technical viability of the STMS approach for variety identification and discrimination will be demonstrated.

In this project, producers of the technology (CPRO-DLO and IPK) are brought together with users, to demonstrate the advantages of the STMS approach to variety identification and its applications. These users are registration authorities in their respective countries (NIAB and CPRO-DLO), a plant breeder (Nunhems) and a service company (Agrogene) that routinely applies the technology for a range of customers throughout the agri production chain.

Applications of molecular marker technology

Identification is important at all stages in the development and production of a variety of an agricultural or horticultural crop. Plant breeders need to be able to distinguish and catalogue their germplasm and to be able to identify readily the more promising breeding lines and traits. In addition, they have a requirement for quickly and reliably tracing infringements of Plant Breeders' Rights (PBR). Seed certification, which forms a link between variety registration and seed production, involves an assessment of both varietal identity and purity, to assure the quality of seed marketed to the farmer or grower. Finally, the ultimate purchaser of the harvested seed or product, who could be a food processor, wholesaler or retail consumer, also needs to be certain that they are obtaining the correct and appropriate variety.

Several applications of molecular marker technology can be envisaged for registration authorities:

- Construction of molecular databases of varieties, to be used as a back catalogue
- Selecting reference varieties for PBR tests
- Grouping of existing varieties and candidates, prior to testing
- Distinctness, Uniformity and Stability (DUS) testing
- Establishing criteria for 'essential derivation'

Objectives and work content

In this project, the technical viability of the STMS technique for variety identification will be demonstrated in two important European crops species: tomato (*Lycopersicon esculentum*) and wheat (*Triticum aestivum).* Application of the technique in each species has been reported by Plaschke *et al.* (1995) and Smulders *et al.* (1995) respectively.

A set of approximately 20 STMS primer pairs will be used for each crop. All of the necessary primer pairs are available, as are systems for detecting STMS polymorphisms. The available STMS markers have been previously shown to reveal polymorphisms within a small collection of reference varieties. These STMS markers will now be tested on a larger set of varieties (approximately 500) to evaluate thoroughly the potential of the technique and to demonstrate the superior technical performance of STMS compared to other marker technologies.

The main objective of this project will be to demonstrate that sequence tagged site microsatellites can be used for the identification of varieties in a practical context and in realistic situations. Thus ideally every variety that has been utilised commercially in Europe in the last ten years should be identifiable by a unique STMS genotype. Practically, it would be sufficient if in a pairwise comparison, more than 99% of the varieties are shown to be distinct. In the worst case this means that at least 90% of the varieties need to have a unique pattern. The main risk to the success of this demonstration project is that there is not sufficient polymorphism detected among the varieties or among a subset of varieties using the primer pairs available in the species of choice. When necessary, this risk can be overcome by using more than 20 primer pairs.

Databases containing the molecular description of the most common varieties of tomato and wheat grown during the last 10 years in Europe will be constructed and tested. It will be demonstrated that the markers and databases can be utilised for a range of applications, including all aspects of variety identification, quality control and genetic diversity measurements. In addition, their potential use in variety registration as a grouping character, through DUS testing, quantifying and establishing 'minimum distances' between varieties and setting criteria for the assessment of 'essential derivation' of varieties will be explored.

Methodology

Within the project, it is important to standardise the methodology and the interpretation of the results. Several systems are available for the detection of STMS polymorphisms. These systems differ with respect to their mode of action, the costs involved in setting up the detection system and the type of laboratory facilities needed. Instead of choosing one system to be used by all participants within this project, the participants will utilise the system that is currently in use in their laboratory. Automated DNA sequencers are used by CPRO-DLO (ALFexpress), IPK (ALF) and Nunhems (ABI). NIAB uses autoradiography to detect products labelled by direct incorporation of ^{33}P labelled nucleotides, whilst Agrogene uses a combined fluoro- phosphorimager in conjunction with fluorescently labelled primers. As the first activity within the project, methodology and the interpretation of results were standardised between laboratories utilising these different detection systems.

The participants agreed a standard set of general PCR conditions (reaction volume, quantity of primer in reaction, quantity of nucleotides, type and number of units of enzyme, length of 72°C extension at end of reaction, etc.) plus specific conditions for each primer pair (annealing temperature, lengths of steps, number of cycles, acceptable other primers for multiplexing). Approximately 20 STMS primer pairs were selected for each crop from existing collections, on the basis that they have been previously shown to reveal polymorphisms in small sets of varieties. Additional information about allelic variation, specificity and map position was also taken into account. The 20 selected STMS primer pairs and a number of possible replacements were analysed with a test set of 8 varieties of each species. In general, the STMS patterns generated were relatively easy to score and showed polymorphism among the varieties of the test set. Example electrophoreograms of the wheat microsatellite Taglgap used to analyse the 8 varieties of the test set are shown in Figure 1. In most cases allele scoring by the different partners resulted in the same classification of the varieties. However, allele designation by fragment length in base pairs was not possible. As illustrated in Table 1, there were consistent band sizing differences between laboratories, probably as a result of the different detection systems used and associated differences in acrylamide, size markers etc.

Table 1

Band sizes in bp found for the wheat STMS marker Taglgap.

	Band 1 (null allele)*	Band 2	Band 3	Band 4
Agrogene	0	207	240	285
IPK	0	209	232	281
NIAB	0	214	238	284
CPRO	0	205	230	282

*) The null is found whenever 1BS is deleted (or this partcular part of 1BS). 99.9% of times this is due to the presence of the 1BL.1RS chromosome, i.e. where the short arm of 1B has been replaced by the homoeologous sequence from rye (detected by the secalin marker).

Despite differences in allele sizing it is essential that all laboratories have the ability to call alleles the same. To make this possible, mixtures of the DNAs that produce specific alleles with a specific STMS primer pair will be made. These reference mixtures will be amplified every time samples are analysed. Gels will be run with suitable size markers and the PCR products of the reference mixture. Allele identification will be carried out by comparison with the alleles in the reference mixture. New alleles may be added to the mixture subsequently. All alleles will be identified by codes.

Ring test

A collaborative, inter-laboratory ring test is being conducted to test the methodology and interpretation of the results. All participants will analyse the same collection of varieties of the two crops. This test will be conducted using 32 samples (16 tomato, 16 wheat), which will include some duplicates, and a set of 22 tomato and 27 wheat primer pairs. All laboratories will produce a description for the alleles observed. The gels and allelic description can be used as reference gels in throughout the project. Results of the ring test will be discussed during the next meeting of the project partners and will be presented.

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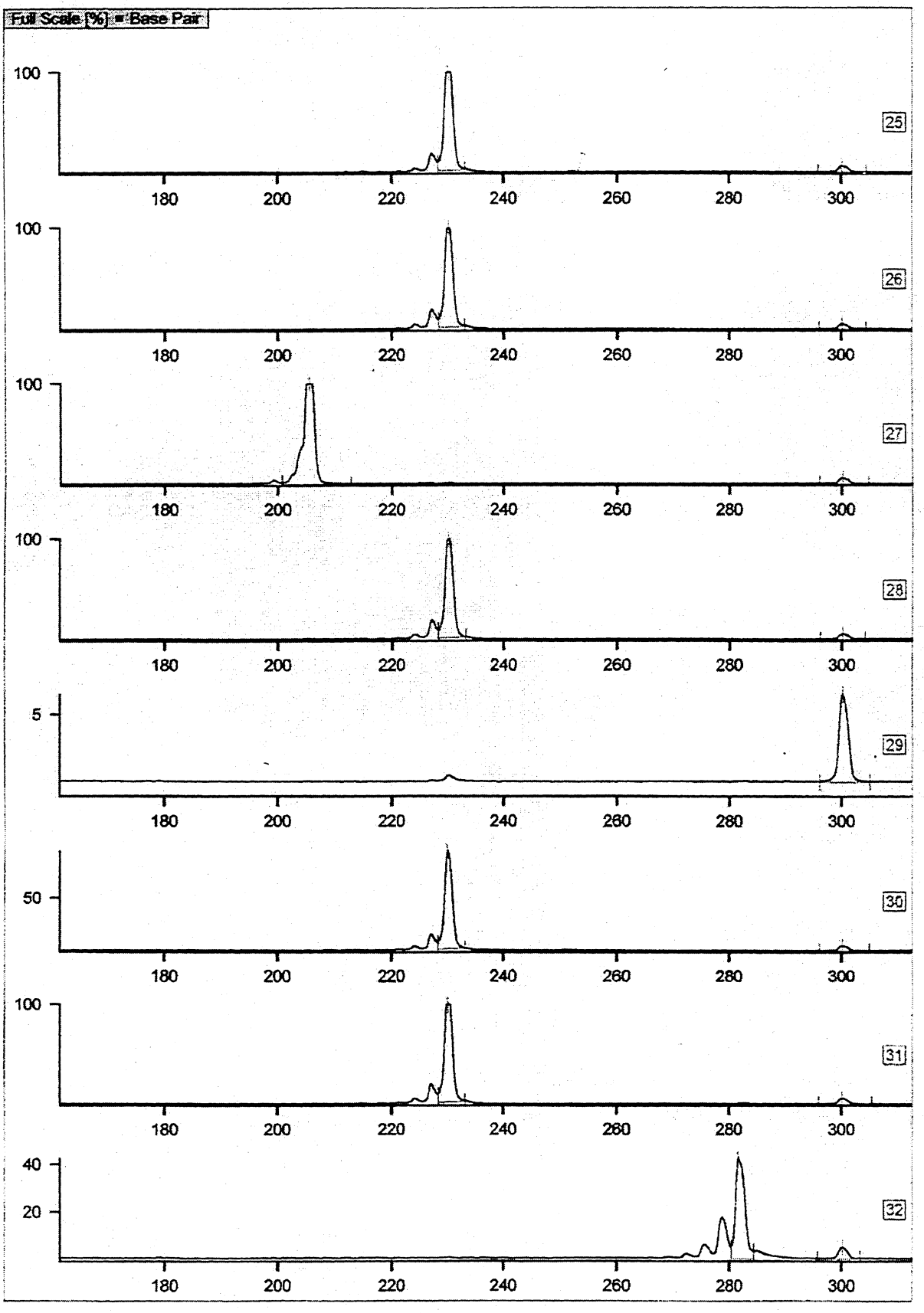
Figure 1



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