FINAL TECHNICAL REPORT

R6951 :

Saturation of the pearl millet genetic maps with molecular markers and fine-mapping of regions of agronomic importance

Executive Summary

Molecular markers and maps are the basis for trait analysis studies and marker-assisted selection. The main objectives of project R6951 were to further saturate the available genetic maps and to produce new, user-friendly markers that could be used to screen breeding materials fast and efficiently. The density of the markers was increased using AFLPs and PCR-based microsatellite markers were generated. These markers were provided to ICRISAT and used for marker-assisted selection to enhance downy mildew resistance in pearl millet hybrids. In addition, a pilot study was carried out to assess the prevalence and use of single nucleotide polymorphism (SNP) as a new marker system for pearl millet. The fact that the first improved variety produced by marker-assisted selection is ready for release to the farmers indicates the success of the pearl millet molecular marker work.

The conservation of colinearity between the genomes of different cereal species can be used to enhance the knowledge on all crops. In order for pear millet to benefit from the comparative knowledge, the relationship between its genome and that of rice was established. This will allow the transfer of information and resources from rice and other grass species to pearl millet, for example to tag regions of the genome controlling drought tolerance.

Background

The application of biotechnology in conventional breeding programs can greatly increase the efficiency and speed with which new varieties are created and released. Molecular marker capability is used in the ICRISAT breeding programmes and is under development in private breeding companies in India. The DFID PSRP programme is the only source, private or public, of molecular markers for pearl millet.

Good restriction fragment length polymorphism (RFLP) maps were available for pearl millet at the start of the project. However, due to unequal distribution of recombination, gaps were present in the distal regions of the millet map, which may result in QTL for traits remaining undetected. Further markers for these regions were needed. In addition, RFLP markers are cumbersome and time-consuming to use. Conversion of RFLP markers to PCR-based sequence-tagged-sites (STS) had provided easy-to-use markers albeit with low polymorphism levels. There was therefore a clear need for user-friendly markers that were able to detect high levels of variation. Microsatellite markers have been developed in a range of crop species. Because they are PCR-based, they are mostly species-specific and non-transferable across crops. Pearl millet thus cannot take advantage of the existing microsatellite markers and a set of pearl millet microsatellite markers needed to be developed.

Comparative genetic mapping within the grass crops has shown that gene orders are highly conserved over evolution. This allowed exploitation of resources and information across grass crops. Knowledge of the genome relationships between pearl millet and other

grasses was identified as an important objective, as it would greatly benefit trait analysis in pearl millet.

Project Purpose

The purpose of the project was to generate genetic maps with increased marker density. The maps and markers have been made available to breeders to apply marker-assisted selection (MAS) to their breeding programmes. Use of MAS results in a more efficient incorporation of traits, such as downy mildew resistance and drought tolerance, into elite germplasm. Cultivation of the improved hybrids will ultimately benefit the pearl millet farmers from the arid and semi-arid tropics.

Research Activities

1. AFLP analysis
In an initial study, amplified fragment length polymorphism (AFLP) markers were used in combination with bulked segregant analysis (BSA) to target a gap on pearl millet linkage group (LG) 4. Despite the use of a large number of primer combinations, only one marker was identified, supporting the hypothesis that the gaps are caused by high levels of recombination and may represent physically small regions. Subsequently, random AFLP mapping was used to saturate the maps. Eighty-five AFLP markers were added to the RFLP map, four of which mapped into gaps. To facilitate the transfer of AFLP markers across varieties, AFLP profiles were generated in a panel of 20 varieties and deposited in the database MilletGenes. The AFLP technique was adapted for detection by silverstaining, to promote uptake by developing countries.

2. Development of microsatellite markers
Small-insert genomic libraries, enriched for microsatellites or simple sequence repeats (SSRs), were generated. Recombinant clones were sequenced and analysed for the presence of microsatellite motifs. Primers were generated to the regions flanking the SSRs and used to amplify the markers from genomic DNA from a panel of 20 pearl millet varieties. Microsatellite sequences were also isolated from pearl millet bacterial artificial (BAC) clones. This has the added advantage that mapping of these microsatellites will anchor the BACs to the genetic map, and aid in the construction of a pearl millet physical map. In total, 98 SSR markers have been developed and their profiles deposited in MilletGenes. Seventy-five microsatellites displayed polymorphisms between the parents of the mapping population(s) and have been mapped.

3. Comparative genetic mapping
RFLP probes previously mapped in other grass species were incorporated into the pearl millet genetic map while pearl millet markers were mapped in foxtail millet. This allowed the establishment of the relationship of the pearl millet genome with that of other grass species. The relationship was unexpectedly complex. Despite the close taxonomic relationship between pearl millet and foxtail millet, the pearl millet genome was highly rearranged relative to that of foxtail millet and rice. Nevertheless, colinear regions could be identified. The comparative data has been exploited as a source of markers for trait mapping in pearl millet. For example, markers associated with QTL for drought resistance in rice have been transferred to pearl millet and correspondence between QTL conferring drought resistance in rice and pearl millet is under investigation.

4. Fine-mapping of regions on LGs 1 and 4 carrying genes conferring resistance to downy mildew

This part of the project is being carried out with funding from the European Union (EU) and John Innes Foundation (JIF).

LG 1

A BC_3F_1 line, carrying a segment of P7-3 at the top of LG 1 in a background of 843B, had been produced by ICRISAT, and BC_4F_1 and BC_3F_2 seeds of this line were provided to JIC. P7-3 had previously been shown to carry a QTL for resistance to downy mildew isolates from Patancheru at the top of LG 1, while 843B is susceptible to the disease. A BC_4F_1 line, heterozygous P7-3/843B at the top of LG 1 was selfed to produce a mapping population of more than 5000 seeds. A subset of this population (135 plants) was screened with *Sclerospora graminicola* isolates from Patancheru in the glasshouse at CAZS, Bangor. Downy mildew resistance segregated as a single Mendelian gene. AFLP markers closely flanking the resistance gene have been identified, and work is currently underway to fine-map the resistance gene.

LG 4

Three QTL conferring resistance to downy mildew isolates from Niger had been identified by ICRISAT in collaboration with CAZS, Bangor, in an F_2 population derived from the cross PT 732B x P1449-2. F_3 plants that were heterozygous for a single resistance QTL were selfed to produce mapping populations. Mapping of a downy mildew resistance gene on LG 4 is underway.

5. Mapping of markers potentially associated with disease resistance

Resistance genes are typically characterised by specific motifs such as leucine-rich repeats. Genes carrying these motifs have been isolated from a range of species including rice and barley (D. Leister, Sainsbury Laboratory) and pearl millet (S. Sivaramakrishnan, ICRISAT). These resistance gene homologues were tested for their ability to detect polymorphism between the parents of the pearl millet mapping populations 81B x ICMP 451 and LGD-1-B-10 x ICMP 85410. The rice and barley clones gave no or very poor hybridisation signals. The two pearl millet clones were polymorphic and were mapped.

6. Capping the ends of the maps

Putative telomere-associated sequences were isolated using a PCR approach. Genomic DNA, digested with MseI and ligated to adaptors was amplified using an adapter primer and a set of anchored telomere primers, containing the motif TTTAGGG. Amplified fragments were separated on a denaturing polyacrylamide gel and visualised by silverstaining. A selection of fragments was cut out from the gel, cloned and sequenced. Primers were developed against the obtained sequences, and markers that were polymorphic in the mapping population(s) were mapped. So far, two fragments were identified that capped a linkage group. Four fragments mapped to interstitial locations.

7. Pilot study for the identification of single nucleotide polymorphism (SNP) markers

Single nucleotide polymorphism markers have the advantage that they can be found almost everywhere in the genome, and are suitable for high throughput as well as low cost detection. In a pilot study, the region flanking 16 microsatellite markers was sequenced from five pearl

millet lines and from bulked DNA from 20 lines. Twenty other genomic regions were also evaluated over the six samples. A total of 98 candidate SNPs were identified.

Random sequencing of pearl millet genomic DNA also proved an efficient way to identify microsatellite sequences. Using stringent selection criteria, about one microsatellite per 10 kb was identified. The number of useful polymorphic sequences is likely to increase as other motifs, such as minisatellite-like sequences are taken into account.

8. Development of a transformation system in pearl millet (subcontracted to V. Patell, Avestha Gengraine Technologies, Bangalore)
Scutella of the inbred line 843B served as explants to give rise to embryogenic calli. In first instance, conditions for tissue culture and plant regeneration were optimised. Proliferating calli were transformed using the PDS-1000 Helium gun (BioRad) with GUS and MnSOD constructs. The GUS construct contained the *Hpg* gene that confers hygromycine resistance as a selectable marker. Transformed calli were analysed by PCR for the presence of the GUS and MnSOD genes.

Outputs

1.1. Eighty-five AFLP markers were added to the RFLP map, four of which mapped into gaps.
1.2. AFLP profiles were generated in a panel of 20 varieties and deposited in the database MilletGenes.
1.3. The AFLP technique was adapted for detection by silverstaining, to promote uptake by developing countries.

2.1. Ninety-eight SSR markers have been developed and their profiles deposited in MilletGenes.
2.2. Seventy-five microsatellites displayed polymorphisms between the parents of the mapping population(s) and have been mapped.

3. A comparative genetic map between the genomes of pearl millet, foxtail millet and rice has been constructed.

4. Fine-mapping of downy mildew resistance QTL, effective against Indian and African *Sclerospora graminicola* populations, is underway.

5. Resistance gene homologues from barley and rice were tested in pearl millet but displayed poor hybridisation. Two available resistance genes homologues, isolated from pearl millet, were mapped.

6. A total of 87 putative telomere-associated sequences have been cloned. So far, six of these fragments have sequenced. Two mapped at the end of linkage groups while four mapped interstitially.

7.1. About 200 kb of pearl millet genomic sequence has been obtained.
7.2. A total of 98 putative SNPs have been identified in a sample of 36 markers.
7.3. Using stringent selection criteria, 20 microsatellites have been identified in 200 kb of genomic sequence.

8. The presence of the GUS and MnSOD genes was shown in transformed calli.

A further objective, to determine the correspondence between linkage groups and karyotype was initiated, but not completed. This was due to the limited resource put into that aspect of the project, as the main focus of the project had shifted to the development of microsatellite markers.

Contribution of Outputs

In the current ICRISAT Medium Term Plan, research into the control of terminal drought and of downy mildew infestations were set as the highest priority. The pay-off from the research for resolving these biotic and abiotic constraints is an estimated $40 million for drought, $15.5 million for seedling thermotolerance and $75 million for downy mildew resistance. Research programmes at IGER, Aberystwyth, the University of Wales, Bangor, and ICRISAT, Patancheru, are directed towards the identification and location of QTL associated with these traits, and the results are implemented at ICRISAT. The molecular markers and maps form the basis of this research. Information on the available materials has been distributed through the pearl millet database, MilletGenes, and a number of non-electronic communication means, such as journals, newsletters, conference proceedings, *etc*. The pearl millet groups at Bangor, Aberystwyth and ICRISAT have direct access to the workstation at JIC. MilletGenes is also available on the worldwide web.

All outputs of the research, including those obtained in collaboration with private companies, remain in the public domain. All probes and primers are freely available to both the scientific community and private breeding companies. The success of the project can be gauged from the received requests for markers, both RFLP and microsatellite markers. All microsatellite markers have been made available to ICRISAT. In addition, SSRs were supplied to millet groups in the UK, France, Japan, USA and Zimbabwe.