WORKSHOP CONCLUSIONS AND RECOMMENDATIONS

Session 1. Introduction and Application of New Technologies in Plant Breeding

Introduction

- Progress in breeding will be accelerated if breeders can integrate their activities with those of scientists working in other disciplines. The purpose of this Workshop was to bring together traditional breeders and experts in molecular biology to stimulate discussions on the potential of new technologies in cocoa breeding.
- There is much to learn from research in other crops, where productivity has been dramatically increased, 50% by genetic improvement and 50% by agronomic measures. In view of the low overall increase in cocoa productivity, there is a lot to be gained through genetic improvement.
- Efforts in traditional breeding need to be maintained and strengthened; if this does not happen there will be no good platform to benefit from the introduction of new technologies. Effective collaboration between traditional breeders and biotechnologists is required to strengthen cocoa breeding as a whole.

Management of genetic diversity

- Molecular markers can be effectively used to verify mislabelling (SSRs, CAPs, SCARs), to evaluate genetic diversity and develop core collections (AFLPs, ISSRs and SSRs), and to search for candidate genes in germplasm collections (gene-specific PCR).

Marker assisted breeding

- Selection efficiency can be improved through the use of DNA markers associated with QTL or with candidate genes, particularly in introgressive breeding strategies.
- Replicated progenies, made up of a large number of individuals and planted at different sites, are required to take full advantage of QTL analysis. These should enable minor QTLs to be mapped and the stability across environments to be verified.

Genetic modification

- Although no commercially grown cocoa has been genetically modified, there has been a ten-fold increase in the acreage of other genetically modified crops over the last three years. Although the public is gradually becoming more aware of GM technology and its benefits, it should be noted that in a significant proportion of the chocolate consuming world, current consumer preference is for non-GMO products.
- In other crops, genetic modification has sometimes been targeted at benefiting the farmer, and in others, the consumer. Currently most applications relate to the correction of only those genetic weaknesses of the crop that are controlled by one or a few genes (*e.g.* susceptibility to pests, diseases or stress conditions).
- Traditional breeding will continue to be very important to create improved populations and to handle traits determined by several genes.

- There is a trend towards the introduction of more than one gene into genetically modified varieties through the use of tissue specific and inducible promoter sequences.
- An efficient genetic transformation system in cocoa is required for research and significant breeding perspectives in the long term future. However, it is essential that any such work is carried out in conjunction with appropriate studies of the impact of the genetically modified organism on the environment and with due consideration to consumer opinion.

Synteny mapping and genome sequencing

- The application of new technologies in cocoa breeding can benefit from the advances made for other crops through synteny mapping; the use of anchored points on the genome which can be used to relate cocoa genetic maps with the maps of other species.
- There is no urgent need for the comprehensive sequencing of the cocoa genome; it is probably better to use information from model crops (candidate gene strategy).

Collaboration between producing and consuming countries

- It is essential to establish effective collaboration between scientists in producing countries and non-producing countries.
- There is a need to develop low-cost, low-tech methods to enable laboratories in producing countries to carry out their part in collaborative studies and to enhance their capacity for innovative research.

Session 2. Identification and Characterisation of Cocoa Genotypes

Main results obtained to date

- So far, various markers have been used: isozymes, RFLP, RAPD, AFLP, I-SSR and SSR. Protocols for sample collection, shipment and analyses have been developed.
- The use of microsatellites (SSR) is the way forward in the short term for fingerprinting, to provide anchor points for mapping populations and for studies using linkage disequilibrium to investigate origins of stocks and gene flow between populations. Researchers at CIRAD have made much progress in this area and have already developed 69 microsatellite primers.
- The USDA is embarking on a large project to genetically characterise the cocoa accessions held in the genebanks of the Americas using an automated microsatellite analysis system, which has the capacity to analyse 1500 samples/person/year.
- It is expected that 15 well chosen SSR will be sufficient for clone identification and characterisation purposes. However, a much larger number of well-identified SSRs are needed for mapping studies (see below).

Applications in cocoa breeding

- Resolution of mislabelling is a major issue for efficient management and transfer of germplasm, for reliable exchange of information on germplasm accessions, for multi-locational trials and, thus, for any collaborative efforts in cocoa germplasm conservation and utilisation.
- Progress made to date is still very limited in view of the importance of the problem.

Recommendations

- A globally co-ordinated effort is required in the area of identification and characterisation of cocoa genotypes in collections. This should include a ring-test to establish the compatibility of the automated system with gel-based systems for SSR analyses.
- Reliable comparison of results between laboratories and between different visualisation techniques will require the use of a common homozygous control clone, *e.g*. Catongo.
- Additional experiments are needed to refine the techniques. These will include i) the adaptation of the various gel-based systems (including techniques which do not involve the use of radioactivity so that the research institutes holding the genebanks can carry out the analysis themselves), ii) determination of the power of resolution of the technique (through sib-analyses) and iii) determination of the frequency of mutations and null alleles.
- It will be essential to identify a "type" specimen for each clone which can be used as a reference to compare all other accessions with the same name. The "type" tree must be selected by an expert, ideally from the original source genebank. Efforts should be made to ensure that it is safeguarded through careful documentation, labelling and possibly cryopreservation.
- Four different options have been presented, with different roles for the collaborative institutes, and advantages/disadvantages compared to a globally co-ordinated effort on cocoa germplasm identification and characterisation.
- If successful, this would allow the research institutes to carry out their own within accession testing using the same SSR primers and make comparisons with the international "type" fingerprint.
- Molecular marker information should be introduced in a standardised form into international databases.
- Strategies are needed for dealing with the off-types detected following molecular characterisation. Genebank managers will have the responsibility of discarding or assigning an appropriate new name to any genotypes which do not conform with the "type" specimen. This information should be disseminated to the cocoa community through the INGENIC Newsletter, the International Cocoa Germplasm Database and other means.

Session 3. Genetic Diversity Analysis

Results obtained to date

- Various molecular techniques and methods of data analyses have been of value in assessing the genetic structure and diversity of cocoa populations. There may be some advantages in using a variety of markers since they may each reveal different parts of the genome.
- Results obtained in different genetic diversity studies, involving in total more than 1000 cocoa genotypes, were analysed during the workshop and the estimated level of heterozygosity of more than 600 genotypes are presented in these proceedings.
- Studies carried out with RFLP, RAPD and microsatellites at CIRAD Montpellier have shown that cocoa populations differ widely in their levels of diversity and heterozygosity. Upper Amazon Forasteros contain high levels of diversity and medium levels of heterozygosity compared to Lower Amazon populations and to wild French Guiana material, both of which exhibit low levels of diversity and

heterozygosity. 'Ancient' Criollo types also form a very distinct, uniform and homozygous group. These results would suggest that founder effects or refuge areas have played an important role in the evolution of *T. cacao* populations. So called Trinitario and 'modern' Criollo types appear to derive from hybridisation between 'ancient' Criollo and Lower Amazon Amelonado.

- RAPD analyses carried out in Trinidad suggest the existence of sub-groups within the Upper Amazon populations: (LCT-EEN + MO), (PA) and (IMC+NA+AMAZ). Scavina genotypes are very distinct. French Guiana materials show a very different RAPD banding pattern compared to other Forastero types. RFLP analyses carried out by Nestlé also identified genetic affinity among IMC and Pound clones (which are NA and IMC types) and among PA types, and again showed the Scavina clones (SCA 6 and SCA 12) to be very distinct.
- RFLP analyses carried out by Nestlé showed that the original Nacional variety is rather homozygous and very distinct from Forastero and Trinitario types. Molecular analyses appear to confirm that many of the cultivated Ecuador cocoa types derive from hybridisation between pure Nacional and introduced Trinitario types.
- RAPD analyses carried out on the CEPLAC collection in Bahia, Brazil have shown continuous variation among the 270 genotypes analysed. A large degree of variation appears among the Upper Amazon types (mainly Pound collections) and among accessions collected from the wild in Brazil. Lower Amazon Amelonado types (Comun variety) appear to be very closely related and at the extreme of the distribution of Forastero types, nearer to Trinitario types. Scavina types form a distinct group at one extreme of the range of genetic diversity, genetically distant to the Lower Amazon and Trinitario groups, and close to some of the accessions from the Ucayali river in Peru. Several unique RAPD bands were identified in the Scavina clones, indicating their distinctiveness. Cultivated and wild genotypes from Ecuador tend to group together between Trinitario and Scavina types, respectively. As expected, clones of hybrid origin, such as CCN 51, tend to be located between the putative parents on genetic diversity maps.

Applications in cocoa breeding

- Information from molecular studies is very useful in managing diversity in genebanks to establish base/core/working collections, ensuring that collections cover the full range of diversity without overrepresentation of certain types and avoiding duplications. This is particularly important with regard to evaluation. Establishment of small representative core collections allows more extensive and uniform evaluation data sets to be assembled.
- Information on the genetic structure of cocoa populations, such as the level of heterozygosity and genetic diversity, is directly useful in breeding. It can be used to guide population enhancement or population breeding programmes, including reciprocal recurrent selection based on recombinations between heterotic groups.
- The information generated can also be of value in attempts to maximise heterosis, and thus produce superior hybrids, since genetically distinct parental genotypes can be identified.
- Cocoa breeding programmes have started to integrate the new information obtained from molecular marker studies to ensure that the diversity of the germplasm is utilised effectively. However, much information is lacking, particularly for the material held in national genebanks.

Recommendations

- Current findings, using isozymes, RAPD and microsatellite analyses, indicate that a large part of the diversity of cocoa has not yet been exploited in breeding programmes. Collaboration in distributing this germplasm and evaluating its potential through field trials is urgently required.
- Genetic diversity studies need to be continued to give more information on the genetic diversity present in cocoa genebanks, with special emphasis placed on the identification of 'core' collections.
- Continuous collaboration for further evaluation of the level of heterozygosity of important breeding materials and of genetic distance between these clones is required.
- The information on the level of heterozygosity can be used immediately to create new speculative crosses between genetically distinct and homozygous genotypes, which have not yet been used in cocoa breeding (such as crosses between French Guiana, Amelonado, some Upper Amazon clones, Criollo and Nacional types). Such progenies can be expected to be uniform and exhibit good hybrid vigour (as observed in 'single crosses' between improved pure lines in hybrid maize selection). International collaboration is required to create and evaluate such promising 'wide' crosses.
- On the other hand, selection of new clones would be favoured in crosses between heterozygous and genetically distinct genotypes of high agronomic value, facilitating recombination of complementary traits.

Session 4. Correlation of Molecular Markers with Economically Important Traits

Results obtained to date

- QTL for resistance to *Phytophthora* were identified in a collaborative project ('CAOBISCO project'). Co-localisations of QTL were observed for a number of progenies on chromosomes 1, 4 and 9 (coded according to the reference map established at CIRAD).
- One strong QTL for resistance to *Crinipellis* has been identified in SCA 6.
- QTL for agronomic traits such as yield, pod and bean characteristics were identified mainly on chromosomes 1, 4 and 5 in a few different studies. For yield, some variation in QTL was observed over the years. One QTL on chromosome 4 appeared to explain 43% of the phenotypic variation for pod size. A number of co-localisations were observed, mainly for related genotypes, but in some cases also for unrelated genotypes, of Trinitario or Upper Amazon origin. These results suggest a certain stability of QTL in cocoa.
- In one study, a major QTL for general agronomic value was found that explained 27.1% of the total phenotypic variation and was co-localised with a QTL for early flowering and trunk diameter. Pleiotropic and epistatic effects were both detected for these traits.

Applications in cocoa breeding

- The QTL studies have provided useful genetic information on the genetic basis of several selection traits in cocoa. For example, the different QTL identified for *Phytophthora* resistance suggest that breeding approaches allowing for accumulation of different resistance genes could be successful.

- It was generally recognised that the available information is generally not yet sufficient for the direct use of the QTL detected in cocoa breeding.
- After obtaining more robust and stable QTL, the markers associated with the QTL can be used for Marker Assisted Selection (MAS). The technique can be used to accelerate breeding progress, since those plants with QTL for one or more desirable traits could be identified at the seedling stage. It is anticipated that the QTL/MAS systems developed could be transferable for use on similar progenies in other countries.

Recommendations

- A common chromosome identification system for cocoa is essential and it was agreed that the system developed by Lanaud and co-workers at CIRAD should be adopted internationally.
- The identification of QTL should be optimised for a limited number of selection traits. There is a need to choose strong QTL, such as those for significant levels of resistance to several pathogens or strains of the same pathogen. Information from markers can be combined with phenotypic data to obtain a selection index.
- Clonally replicated progenies, containing a large number of individuals, will be needed to take full advantage of the QTL analyses. Each progeny should consist of at least 200 individual plants. It is very important to replicate these progenies in different locations to map minor QTL and verify QTL stability across environments. This can only be achieved through international collaboration.
- Another approach to identify stable QTL is to use the possible linkage disequilibrium that may persist in certain genetically related cocoa populations, such as Trinitario or related Amazon populations (IMC, GU, etc.). Studies have been recently initiated for the Trinitario/Criollo group.
- Further development and transfer of simple marker technology to user countries is required before it can be integrated into cocoa breeding.

Session 5. Other Topics

Resistance gene homology and micro-array consortium

- There are good indications that gene sequences detected in cocoa are similar to known resistance genes in other plant species. Ten putative gene candidates have been identified that probably belong to three families of potential resistance (R) genes. The main objective is to screen germplasm for different resistance alleles.
- Microarrays are miniaturised systems which allow the simultaneous measurement of the comparative expression levels of thousands of genes in experimental and control material. This technique could be used to monitor gene expression profiles during growth and development and in response to biotic and abiotic stresses. This can provide leads to understanding basic molecular mechanisms, for example which pathways are up regulated in response to a pathogen and which are turned off. It also provides a means to rapidly identify candidate genes involved in a target process.
- Microarray systems for cocoa are being set up in several institutions and progress could be accelerated if a cocoa gene expression microarray consortium can be established. A bioinformatics resource base is needed to link data obtained by different research teams.

Applications in CSSV resistance studies and indexing

- Molecular cloning methods have enabled the isolation of full-length infectious clones of severe isolates of CSSV from Togo and Ghana. Mild isolates of the virus, which have potential use in cross-protection, have also been isolated. Infection of cocoa beans and young seedlings by particle bombardment and/or *Agrobacterium*-mediated infection is now possible. With these tools, specific virus inoculum can be quantified in challenging new cultivars in resistance breeding or cross-protection programmes.
- New CSSV-specific primers have been designed for disease indexing by polymerase chain reaction (PCR). Further development of this method is required so that it can form part of a quarantine procedure and thus help prevent the spread of CSSV.

Session 6. Propagation Methods

Somatic embryogenesis (SE)

- SE is a powerful tool for multiplication, germplasm conservation (cryopreservation), germplasm exchange and genetic modification.
- SE technology is not yet ready for commercial scale multiplication of improved cocoa genotypes for farmers usage.
- SE is expensive but can be used for fast multiplication of a limited number of genotypes and the establishment of clonal gardens for further use with conventional propagation methods.
- SE protocols have been developed and the technology applied in at least nine laboratories around the world. Floral parts are the explants of choice.
- The protocols are similar in the use of 2-4D and cytokinin but differ in the use of basal DKW, MS and Woody Plants Media. The majority of the laboratories are using DKW.
- Close to 100 genotypes have been propagated by SE with high efficiency being achieved for a number of genotypes. Conversion was achieved at 55-60%.
- Secondary embryogenesis is more efficient and produces unified embryos.
- SE field tests and DNA tests to verify agronomic value and genetic uniformity are required.
- SE is a potential tool for cocoa germplasm exchange. However, it is not yet known if SE propagated material is guaranteed virus-free (as is the case for zygotic embryos with CSSV). If so, SE could be of great help in speeding up the time involved in transfer of germplasm.

Semi-industrial scale of production of rooted cuttings

- The largest propagation centre 'Biofabrica' was established recently in Bahia, Brazil, using traditional technology adapted from eucalyptus mass propagation systems.
- Currently this centre is propagating 14 cocoa genotypes with resistance to Witches' Broom disease. The rooting house and nurseries have the capacity for a daily production of 50,000 rooted cuttings but at present a shortage of cuttings is restricting the daily production to approximately 10,000 rooted cuttings.