Use of molecular markers for coconut improvement: Status and prospects

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Introduction

Some characteristics of coconut are significant for understanding the constraints and the limitations affecting coconut breeding efficiency:

- It is a tree crop; it has a long life cycle and needs to be observed for several years after sexual maturity, which is reached from 3 to 6 years after field planting. As it is planted at a low density (typically between 140 to 200 palms/ha), genetic trials require large areas.
- It exhibits a large phenotypic diversity from Dwarf to Tall with various intermediate forms. This variability is especially obvious on fruit characters;
- Although Tall cultivars are mainly cross-pollinating, self pollination occurs at a rate that is strongly determined by the environment. Dwarf cultivars are mainly self-pollinated, but not strictly so and Green and Brown Dwarf types often show a certain percentage of cross-pollination;
- It is subject to severe inbreeding depression but may also show high interpopulation heterosis;
- It has a low multiplication rate: in very good conditions it may produce 100 descendants per year only; and
- There is no horticultural vegetative propagation method for coconut and there is no routine *in vitro* vegetative propagation technique.

Seeds for the propagation of coconut are produced by different methods and these include:

- Collecting seednuts in farmers' fields from 'informal' local cultivars;
- Collecting seednuts in research stations or high yielding blocks from 'improved' local cultivars with selected, open-pollinated parents;

- As a variant of the above, collecting seednuts from open-pollinated synthetic variety;
- Seed production in Dwarf x Tall hybrid seed gardens; and
- Seed production in Tall x Tall hybrid seed gardens.

In addition, the trend toward multipurpose uses of coconut is on the increase, which further increases the selection criteria for nut production.

From the above it can be seen that the use of molecular breeding techniques would help in dealing with some of the complexities of coconut breeding. The long life cycle, bulkiness and low rate of multiplication make 'conventional' breeding costly. Identifying molecular markers linked to useful traits and characters is expected to strengthen and speed up breeding, while reducing costs, improving efficiency and reducing the length of the selection cycle. This paper, discusses the different molecular tools that can assist in plant breeding and examines their application to coconut improvement.

Potential for success in molecular breeding in coconut

In general, the use of molecular marker may improve breeding efficiency in different ways: germplasm characterization and management (see Lebrun *et al.*, Chaper 4); linkage mapping and identification of quantitative trait loci (QTL) markers for marker- assisted selection (MAS); identification and physical mapping of genes with known function; and introduction of functional genes into the genome of an individual. Below is a brief discussion on how these different applications could be useful in plant breeding.

Linkage mapping and marker-assisted selection

Dekkers and Hospital (2001) did a comprehensive review of the molecular methods used in the improvement of plants and animals. When ordered on a linkage map, anonymous markers can be used to identify chromosome regions where the QTLs are located. Typically, the precision of the location is about ±10 centimorgans (cM). In principle, there is no functional relationship between the markers used and the actual QTL. They serve only as 'labels' for the QTL: by selecting markers surrounding the favourable allele QTL, this allele is also selected (unless a double recombination occurs in the corresponding interval) and, thus, the desired trait is improved. Various strategies are available to exploit this knowledge: genotype building programmes that consist of assembling the favourable alleles of many QTLs in a single genotype; introgression programmes that are used to introduce the favourable allele of a specific QTL into an otherwise good variety; recurrent selection programmes that

can use molecular score in addition to phenotypic data to predict the value of parents.

Molecular breeding efficiency is at its best with traits exhibiting a moderate heritability, as identifying QTL's markers requires that some genetic variation is observable, while conventional breeding gives satisfactory results with highly heritable traits. Two additional factors are important for the success of MAS compared to conventional breeding. Firstly, close linkage between the markers and the actual QTL is necessary, especially if their association is to survive several recombination cycles. This underlines the need to fine map QTLs wherever possible. Secondly, the higher the percentage of additive genetic variance accounted for by the identified QTL, the higher will be the efficiency of MAS. It must be noted, however, that the efficiency of MAS is often overestimated. This is due to the limited precision of the QTL's location, to its association with unwanted (and unnoticed) unfavourable traits and to epistasis effects or genotype x environment effect. The initial enthusiasm about molecular breeding has thus been tempered with 'cautious optimism'.

As genotyping is still expensive, the cost/benefit ratio of molecular breeding needs to be considered. Situations that are favourable to the use of molecular breeding are mainly those where the target trait is difficult to assess (or cannot be assessed at each breeding cycle) like disease resistance, when the measurement is expensive or time consuming. In the case of coconut, most of the traits related to nut production and quality require several years of observation after sexual maturity. The main expected benefits of MAS in coconut are saving time (by selecting, based on markers only, at an early stage) and space (by combining marker selection in nursery with phenotypic selection on the selected genotypes) in breeding programmes. Both factors are critical for coconut improvement.

Synteny

The above approach requires extensive mapping of the species genome because nothing is assumed about the location of interesting QTLs. Its efficiency may be improved if some information about the location and function of QTLs is available, even from another species. This information may come from a related species, where some QTLs have already been identified. By establishing a correspondence between the two species' chromosome maps (using markers that are polymorphic in both species), it is possible to check in species A for the presence of a QTL that has been identified in species B. Application of this principle has been demonstrated for sorghum and sugarcane (Dufour *et al*. 1997), for rice and sorghum (Ventelon *et al*. 2001) and also for a group of species

including rice, sorghum and maize (Asnaghi *et al*. 2000; Glaszmann *et al*. 1997). The similarities between large fragments of the genome in these three species made it possible to locate a gene for rust resistance in sugarcane. Oil palm is the closest, economically important species, related to coconut. It is anticipated that relatively large parts of the genomes of these species are colinear (i.e. large blocks of genes are ordered in the same way). A total of 915 markers have already been placed on a saturated oil palm linkage map, based on 116 progenies, of which 257 are microsatellites (including 20 markers from coconut), the remaining being AFLPs (amplified fragment-length polymorphism). Twenty four traits, related to yield, quality of oil and vegetative development were observed and about 30 putative QTLs have already been located.

Candidate-gene approach and physical mapping

While synteny exploits the conservation of large portions of the genome in closely related species, the candidate-gene approach exploits the conservation of the genetic structure of functional genes or groups of genes in unrelated species (sometimes, in different reigns). This reflects the fact that the genes, whose function is necessary for the development and for the survival of the organism (i.e. producing a structural protein, an enzyme or a regulating factor), are subject to natural selection. Thus, their sequences are much more conserved than that of non-coding parts of the genome. For this reason, similar genes, with often the same function are found in very distantly related species. Looking for the presence of sequences related to known metabolic functions might be a first step for characterizing potential QTLs and for locating precisely the genes involved. For example, a eukaryotic translation factor is found to be associated to virus resistance in pepper (Ruffel *et al*. in press).

This approach requires knowledge of the fine structure of the genome and a physical mapping of the chromosomal region involved (Han *et al*. 1999). The bacterial artificial chromosomes (BAC) are long DNA sequences that make it possible to construct precise 'physical maps' (i.e., based on sequence length measurement rather on recombination rates). A BAC library of coconut exists at CIRAD and its total length is five times that of the coconut genome. Theoretically, this ensures that a little less of 1% of the coconut genes are not represented in the library. In practice, it may be a little more, as the sequences included in the BACs are not exactly a random sample of the genome.

The possible applications of such an approach in coconut include search for candidates for resistance genes to pathogens. Actually, candidate gene and physical mapping are not breeding methods, but rather tools that can be exploited through MAS or through genetic

engineering. In addition, it should be noted that large mapping populations (about 800 individuals) are necessary to take the full advantage of such approaches.

Genetically-modified organisms

Genetic transformation consists in introducing a gene (from the same species or from another species) into the genome of an organism. After its introduction, this gene functions as a part of the recipient plant genome and may be transmitted sexually. Genetic transformation involves several steps:

- Setting up a genetic construction (a chimerical sequence of DNA), generally including the desired gene a promoter to control the gene expression; and sometimes, a 'selected' gene use for identifying and selecting the transformed plants.
- Introducing the construction into the plant cells (several methods are available).
- Selecting the plants that have included the construction in their genome and thus express the 'selected' gene.
- Checking that the transformed plant behaves as expected.

There are several applications of genetic transformation in plants and may include the following objectives:

- Production of plants adapted to environmental stresses, like drought or resistant to pests or diseases.
- Production of substances with insecticidal properties in its leaves, or by introducing resistance to a herbicide into a plant to help reduce the cost of crop maintenance.
- Alteration of the chemical composition of the product- for example, modified oil composition, with increased lauric acid content for canola, production of medicines.
- Alteration of plant physiology – a possible application in coconut is the introduction of male sterility for making hybrid production easier (Rohde *pers. comm.*).

A review of the use of genetic engineering for food uses in soybean is presented by Kinney (1996). It is considered by some as the only way, food supply can be increased to a level required by the increase in world population (Kasha 1999). Research on the stimulation osmotic adjustment (accumulation of non-toxic compound in plant cells under water stress condition) has been actively conducted in the last 20 years. At the cell level, this accumulation is expected to reduce osmotic pressure and thus maintain water absorption and cell turgor pressure, which might contribute to sustaining physiological processes, such as stomatal opening.

At the plant level, it is expected to lead to increased yield. The physiological and genetic bases of this phenomenon are presented in Zhang *et al*. (1999), along with breeding strategies.

However, the relevance of this mechanism is questioned by Serraj and Sinclair (2002). Their main argument is that, overall, increased crop yields require increased water consumption due to photosynthesis. As a result, such a mechanism is likely to favour water conservation (and thus survival) rather than increased yield. In effect, a review of experimental results shows that favourable effect of osmotic adjustment is mainly observed in extreme drought conditions where yields are very low in all treatments. Such conditions are not compatible with profitable agriculture. These authors suggest that such a mechanism could be useful, only in the case where the maintenance of rood development makes it possible to reach deeper underground water.

Research activities in molecular genetics conducted worldwide related to coconut improvement

Molecular genetics research in coconut has developed significantly since the beginning of the 90's in several fields. The preliminary step was to devise suitable markers and to use them for assessing the genetic diversity in coconut. Once these tools have been developed, they were used primarily for studying the coconut genetic diversity. One of the applications of such studies was the creation of a molecular kit for identifying coconut cultivars. The second potential use was to prepare for marker- assisted selection in coconut. It then became necessary to produce linkage maps.

Devising markers for coconuts

According to their availability in the different laboratories and to convenience, a variety of methods has been used:

- RAPD (Random amplification of polymorphic DNA: Ashburner *et al*. 1997; Duran *et al*. 1997; Wadt *et al*. 1999)
- RFLP (Restriction fragment length polymorphism: Lebrun *et al*. 1999; Lebrun *et al*. 1998)
- AFLP (Amplified fragment-length polymorphism: Perera *et al*. 1998)
- ISTR (Inverse sequence-tagged repeat: Duran *et al*. 1997; Rohde *et al*. 2000)
- Microsatellites (Duran *et al*. 1997; Karp 1999; Perera *et al*. 1999; Rivera *et al*. 1999)

See article on biochemical and molecular methods in Chapter 4 for more details.

Linkage mapping and QTL identification

Two coconut linkage maps have been constructed. The first one was made in the Philippines using hybrid seedlings of a Malayan Yellow Dwarf (MYD) x Laguna Tall (LAGT). A total of 382 markers were placed on 16 linkage groups. Six QTLs for early flowering were identified (Herran *et al*. 2000). The second map was a collective work based on a MYD x Rennell Island Tall planted in Côte d'Ivoire. A total of 227 markers were arranged in 16 linkage groups. Nine QTLs related to fruit number were identified (Lebrun *et al*. 2001). Both of these studies assigned a total length of about 2000cM to the coconut genome. Two other mapping populations; East African Tall (EAT) x Pemba Red Dwarf (PRD) and EAT x RIT in Tanzania are under study in the framework of a European joint research project on oil palm and coconut (INCO LINK2PALM project). More phenotypic observations are being made on these mapping populations in order to identify QTLs. Under INCO LINK2PALM, it is planned to increase the number of markers on the maps. The inclusion of common markers will help the construction of a high-density reference map, which will be useful for further studies.

Devising adapted mapping populations for QTL identification

Although F_1 hybrids have two parents, recombination occurs only between genes from the same parent. Therefore, the usefulness of linkage maps based on this type of hybrids is limited because they exploit only a part of the existing genetic diversity that is related to within-cultivar polymorphism. Moreover, the chances of observing segregation at the same QTL in another cross are less than 50%. Linkage maps based on second generation hybrids do not have these drawbacks. As shown by several studies (see article on biochemical and molecular methods in Chapter 4), a large part of the genetic diversity in *Cocos nucifera* L. occurs between the two major cultivar groups, namely the Pacific group and the Indo-Atlantic group. Thus, a special crossing plan involving second generation hybrids has been devised to identify the QTLs that account for the differences between the two groups (Figure 1). Using such a design will result in more QTLs identified through the choice of genetically distant parental populations. Moreover, it will be easier to use these QTLs in practical breeding because the emphasis will be on the differences between cultivar groups and not on the variation within a cultivar.

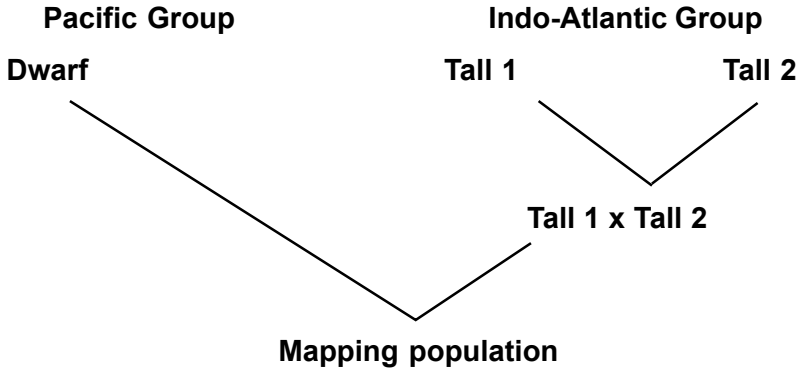

Figure 1. A genetic design adapted to identifying QTLs in coconut.

The use of a well chosen set of Dwarf mother palms as a tester makes it possible to produce a large number of progenies while simplifying the mapping task due to its highly homozygous genetic structure. Such a mapping population is being planted in the Philippines in the framework of the INCO-LINK2PALM project. Another population is available in Côte d'Ivoire.

Results and evidence of the success of MAS in coconut

Usefulness of various markers

As noted earlier, a large number of markers of different types have been designed for coconut. Among them, microsatellites markers are considered as the most useful because they are codominant and highly polymorphic; they give repeatable results, using a non-radioactive PCR technique; and it is not too demanding in terms of DNA quantity or quality. However, other markers, like AFLPs may be useful, particularly for constructing high-density linkage maps. Such maps are useful for two reasons: the closer the mapping of a QTL, the smaller is the probability of a double crossing over between the surrounding markers. Such an event results in selecting plants that have the markers, but not the QTL itself. Although, normally, such an event is relatively rare, it may become a real nuisance in selection programmes that spread over several generations. The second reason is that the region that contain the QTL also contain many other genes, which are not necessary favourable.

Linkage mapping

The available results in linkage mapping demonstrate the feasibility of constructing a map with reasonably good coverage of the coconut genome,

thus obtaining 16 linkage groups. Some QTLs for traits related to early development and production have also been identified. The work that is underway through the collaboration of several research teams in the framework of the INCO LINK2PALM project will make it possible to increase the density of markers and also identify more QTL markers in coconut.

In the next few years, another generation of mapping population will be made available for molecular breeding. In these populations, QTLs related to between-cultivar differences rather than on within-cultivar variation is being emphasized. It is expected that such QTLs will be much easier to use in coconut breeding than those located in presently available mapping populations because they correspond to differences between cultivars (and possibly, between molecular groups) rather than differences between individuals of the same cultivar. Their utilization will not be restricted to the exploitation of a single cross.

Prospects of GMOs to date

There has been little progress on developing genetically modified coconuts. The most obvious constraint is the lack of efficient method of plant regeneration method. Other constraints range from the limited amount of research personnel allocated to molecular genetics in coconut to the lack of long-term visibility of the benefit for the planters. For example, modifying the coconut genome to produce high market-value product could increase the planter's revenue, but also make this revenue very dependent on the fluctuations of the market. Such a strategy is easier to justify in an annual crop.

Immediate research needs for using molecular biological techniques for coconut improvement

In the framework of a global programme for coconut, one of the main objectives of molecular breeding research efforts would be to obtain information on the location of important QTLs on the coconut genetic map. As suggested above, such a result can be obtained by exploiting the progenies of wide crosses such as West African Tall x Rennell Island Tall. This objective implies the availability of suitable mapping populations, constructing the corresponding linkage map and performing the necessary observations in the field, in order to correlate phenotypic data and locations on the map. Below are some important elements of research that are required for the successful use of molecular biological techniques in coconut improvement.

Planting and studying suitable mapping populations with molecular markers

Populations corresponding to the genetic structure represented in Figure 1 already exist in Côte d'Ivoire and are being planted in the Philippines. In the case of the Philippines, financing was secured for performing crosses and planting through the INCO LINK2PALM project. Once the population is available, constructing a linkage map involves observing from 300 to 400 markers on 150 to 200 individuals.

Obtaining a large number of good quality observations on mapping population

Molecular markers studies are not a goal by themselves. The benefit from such studies can only be obtained by performing a large number of observations on each of the individuals of the mapping population. This includes traits related to yield and quality of the product, vegetative traits that may be important for adaptation to various environments or to cultivation conditions. A last category corresponds to physiological traits such as photosynthetic efficiency that may be relevant to explaining a genotype's performance.

The resistance to pest and diseases was not included in the above list for the following reasons: assessing resistance is possible only in the presence of the pathogen, which might affect the other traits in an unpredictable way and breeding for resistance requires specific experimental designs. Moreover, for the major diseases affecting coconut, efficient methods for artificially inoculating the palms to create epiphytotic conditions disease screening are lacking and the fate of such resistance trials depends on the rather unpredictable transmission of the disease. Even when the disease is present, the question remains, whether a healthy palm is truly resistant or, simply, was not contaminated.

Reliable small-scale vegetative propagation

Until inoculation methods are set up, small-scale vegetative propagation could help cope with this difficulty. However, in contrast with what was once expected from *in vitro* culture (i.e. production of tens of thousand of plantlets from a single adult palm), the objective has rather been to produce ten to twenty plantlets from about 200 embryos with a 80% success rate. When it becomes feasible to produce significant number of palms through tissue culture, it could help assess resistance to disease, by planting the seedlings produced following an appropriate statistical design. Hence, there is a need for increased research on tissue culture and somatic embryogenesis to be able to produce enough seedlings required for various tests that molecular breeding would demand.

Coconut and genomics

As more and more tools are being developed for locating precisely potential genes of interest, the potential benefits of candidate-gene approach and of physical mapping should be considered. This requires a careful analysis of costs and of potential benefits. Such approaches generally require a very good knowledge of the trait physiology and validating a candidate gene as being the gene of interest requires high resolution linkage analysis in large populations (Pflieger *et al*. 2001) and thus, costs time and money. With regard to disease resistance breeding, the lack of reliable methods for assessing the resistance of individual genotype is a serious limiting factor. Thus, there is a need for serious strategic reflection on genomics in coconut.

Constraints and opportunities

Constraints

Coconut breeding suffers mainly from a severely limited amount of resources, both financial and human. The characteristics of the crop make breeding both slow and costly. It takes at least 14 years for a generation and one hectare for testing each hybrid. Very few coconut-producing countries have the necessary resources for a comprehensive programme and coconut breeding has often been limited to testing a few F_1 hybrids between populations.

Once good hybrids and varieties are selected, the difficulty is to reproduce them and to make seednuts available to farmers. In most producer countries, hybrid seed production has been the main obstacle in extending the hybrids under cultivation. The lack of an efficient vegetative propagation method also makes it difficult to take advantage of breeding progress.

Opportunities

First with the creation of the International Coconut Genetic Resources Network (COGENT), and now with the establishment of a Global Coconut Research for Development Programme (PROCORD), there is an increasing recognition of the coconut as a strategic crop for many tropical countries. Within this framework, coconut-producing countries as well as countries interested to assist them can collaborate, working in a network, for a globally-coordinated coconut breeding programme. Each country can participate in the activities in which it is most interested, within its available resources. Using molecular techniques makes the programme even more attractive. The network may involve laboratories and research teams from developing and developed countries. It can also use other possibilities such as synteny with oil palm.

It must be noted, however, that the use of molecular markers may facilitate breeding but does not replace conventional breeding. The efficiency of marker-assisted breeding still depends on the quality and accuracy of field observations and experimentation.

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