Chapter 6
Coconut Biotechnology

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Abstract The major hurdles for genetic improvement of coconut are attributed to its perennial nature, long juvenile period and high heterozygosity, which make conventional coconut breeding time-consuming, resource demanding and laborious. Biotechnological tools have permitted researchers to overcome some of these impediments. Embryo culture has been successfully utilised for international germplasm exchange. Cryopreservation techniques have been standardised for zygotic embryos and pollen, which enable safe and long-term conservation of coconut genetic resources. A repertoire of molecular markers has been utilised for comprehending and exploiting the genetic diversity existing among coconut populations world-wide. Molecular markers have also been employed to generate linkage map for QTLs involving yield attributes. The limited availability of genomic resources/information, a restraint to genomics-assisted crop improvement in coconut, is gradually being surmounted with the research on coconut genomics and transcriptomics gaining impetus during the last few years. This chapter amply elaborates the efforts that have been instilled and the success accomplished with respect to coconut biotechnology.

6.1 Introduction

Conventional genetic analysis has been difficult in coconut, given its perennial nature, extended juvenile period, long breeding and selection cycle, difficulties in raising F_2 progenies and high inherent heterozygosity, especially of tall cultivars. Therefore, genetics of most of the agronomically important traits have been

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inadequately investigated in coconut. These intrinsic limitations in traditional coconut breeding have made the prospects of exploitation of biotechnological tools attractive.

Non-availability of quality planting materials has been a major constraint in coconut productivity. Since the palm has a single apical meristem, without branches or suckers, its propagation is restricted to seed propagation exclusively. Without any other known method of propagating the coconut palm through vegetative means, much hope was placed on use of tissue culture to vegetatively propagate high yielding and disease-free coconut genotypes. In spite of concerted efforts in a number of laboratories world-wide, a commercial in vitro regeneration protocol has not been standardised till date. The difficulties of cloning coconut through tissue culture have been addressed in a number of research laboratories around the globe since the late 1970s. Many of the earlier efforts were concentrated on procedures for reversion of floral meristem into vegetative ones; the lack of success in these initiatives diverted research efforts to induction of somatic embryos from a variety of explants. Unfortunately, success has been quite limited due to a number of limitations (Fernando et al. 2010). The frequency of regeneration obtained has been quite low, and only a small number of tissue culture-raised plantlets have been successfully established in the field. The success obtained in embryo culture and its use in germplasm collection and exchange and also embryo rescue have been the major achievements with regard to use of tissue culture techniques in coconut.

In vitro conservation has been used as an adjunct approach to facilitate conservation of coconut genetic resources and ease safe international exchange of germplasm. These techniques need minimum space for storage of vast germplasm collections and enable supply of valuable genetic resources. Success in in vitro conservation of coconut genetic resources has been achieved by cryopreservation of zygotic embryo, pollen and plumular tissues.

Application of molecular markers in coconut commenced in the early 1990s, and their uses have been varied, ranging from assessment of genetic diversity to generating linkage maps. Initial investigations were focused on evaluation of coconut genetic diversity and genetic relatedness at the protein and isozyme levels and afterwards at the DNA level using a gamut of molecular markers, with many of the recent research utilising the codominant microsatellites for characterisation of germplasm, for hybridity testing and for construction of genetic linkage maps.

Knowledge derived from recent genome and transcriptome studies will facilitate discovery of candidate genes that govern key developmental and agronomical traits in coconut, in addition to providing important resources for comparative genomic studies in Arecaceae.

This chapter comprehensively covers the major developments in coconut biotechnology during the past few decades emphasising tissue culture methodologies and their applications, molecular markers, gene discovery and, finally, genomics and transcriptomics.

6.2 In Vitro Techniques

6.2.1 Embryo Culture

6.2.1.1 Germplasm Collection

The natural distribution and transcontinental dispersal across land/sea routes since time immemorial and scientific collection and exchange of coconut genetic resources have solely been based on seed nuts since they represent the only planting material for propagation in coconut with no means of vegetative propagation. However, the bulky nature of the seed nuts, its short dormancy period, presence of nut water, stringent phytosanitary requirements and high cost for transportation are major impediments encountered during the course of germplasm collection and storage. Therefore, according to the technical guidelines of the Bioversity International, collection and transportation of coconut for the safe movement of coconut germplasm is advocated through embryo culture (Diekmann 1997). Besides overcoming formalities of quarantine regulations which include treatments of the nuts with chemicals, collection as embryos also lessens the cost of transport to a great extent, and therefore coconut zygotic embryo culture has immense practical value with respect to collection and exchange of germplasm between countries.

Though many efforts in standardising zygotic embryo culture have been reported in the past (Cutter and Wilson 1954; Abraham and Thomas 1962; Ventura et al. 1966), the ICAR-CPCRI developed for the first time a successful procedure for culturing coconut zygotic embryos from 8- to 11-month-old nuts, which has been effectively utilised in the germplasm expeditions of the institute since then (Karun et al. 1999, 2004). The protocol utilises an effective artificial medium which sustains the extracted embryo to grow and to form entire plantlets under in vitro conditions. The field collection technique includes inoculation of the sterilised zygotic embryos, excised from the nuts, on to the nutrient medium in vitro (Assy-Bah et al. 1987; Sossou et al. 1987; Karunaratne 1988; Rillo and Paloma 1991; Karun et al. 1993; Ashburner et al. 1995). Storage of zygotic embryos is essential when the collection sites are remote. Assy-Bah et al. (1987) reported that endosperm plugs, scooped from mature nuts, could be stored in KCl solution for up to 2 weeks. In a later study, Karun and Sajini (1994) established that sterile water could also be made use of as medium for storing zygotic embryos for 2 months; this approach has been utilised in all the germplasm collection expeditions carried out by the ICAR-CPCRI. Germination of embryos was inhibited in media free of sucrose and activated charcoal (De Guzman et al. 1971; Karunaratne et al. 1985; Assy-Bah et al. 1987; Rillo and Paloma 1990).

The embryo culture protocol developed at the Central Plantation Crops Research Institute under the Indian Council of Agricultural Research (ICAR-CPCRI) by Karun et al. (1993) was successfully employed for the first time during 1994 for transferring six Pacific Ocean accessions, maintained at the World Coconut Germplasm Centre, Andaman Islands, to the mainland. Afterwards, 5 international

expeditions were conducted by the institute during 1997–2001 for the collection of coconut genetic diversity (Karun et al. 2002), wherein a total of 4182 embryos of 45 accessions were collected from 8 countries (Mauritius, Madagascar, Seychelles, Maldives, Comoros, Reunion, Sri Lanka and Bangladesh). Variations were recorded for the per cent retrieval of embryos among locations and accessions with the percentage of germination varying between 54 (Sri Lanka) and 82.2 (Bangladesh). All the exotic accession collections made as embryo cultures have been planted in the International Coconut Genebank (ICG-SA) and have commenced flowering since 2004. From the results obtained from studies on in vitro retrieval of embryos and their ex vitro establishment, it has been concluded that about 300–400 embryos (i.e. three to four times the actual requirement) are needed for successful establishment of 100 palms in a field gene bank. The diverse collections, after evaluation, are being exploited for evolving new varieties in coconut.

6.2.1.2 Embryo Rescue

Embryo culture technique has been successfully utilised in coconut for obtaining plantlets from embryos which fail to germinate naturally (De Guzman et al. 1971; Gosal and Bajaj 1983; Thomas and Pratt 1981). A commercial application of embryo culture has been the rescue and culture of Makapuno variety, with jelly-like endosperm, in the Philippines (De Guzman 1970; De Guzman et al. 1971; Del Rosario and De Guzman 1976). Successful embryo rescue of a similar variety from Sri Lanka (‘Dikiri Pol’) has been also achieved (Vidhanaarachi et al. 1998). In India, embryos from sweet kernelled nuts from the Konkan region of Maharashtra have been excised out and plantlets regenerated successfully through the embryo culture protocol (CPCRI 2011). Horned coconut from Andaman Islands, India, produces multiple ovaries which result in development of hornlike structures over the mature fruits, thus delaying germination. Such embryos were cultured in vitro, and plantlets could be retrieved using embryo culture technique. Field evaluation of these plantlets has revealed that the trait is inherited (CPCRI 2012).

6.2.2 Cryopreservation

Palm genetic resources are traditionally conserved ex situ as whole plants in field gene banks which require large area, besides huge investments in terms of financial, infrastructural and manpower resources. Cryopreservation of coconut pollen and embryo is an excellent option for long-term conservation of genetic resources, which can provide a viable backup to field gene banks. Coconut, being a recalcitrant crop, is sensitive to desiccation and can thus be conserved only for short periods even under optimal moisture conditions (Assy-Bah and Engelmann 1992b). This necessitates pretreatments before conserving in liquid nitrogen at −196 °C; pretreatments include simple desiccation (using laminar air current and use of silica

gel), the use of high concentration of sucrose, various cryoprotectants in various combinations (glycerol, propylene glycol, DMSO, sorbitol, formamide) and encapsulation with 3% sodium alginate and dehydration techniques.

6.2.2.1 Embryo Cryopreservation

Bajaj (1984) proposed the prospects of long-term conservation of coconut zygotic embryos when the embryos resumed growth after freezing at −196 °C. In the experiments carried out, immature embryos of West Coast Tall cultivar were partially dehydrated and cut into transverse halves, treated with a cryoprotectant solution (7% DMSO and 7% sucrose in MS liquid medium), blotted dry and wrapped in a single layer of sterile aluminium foil. It was then frozen by gradually lowering into liquid nitrogen and kept for 5 min. The frozen samples were thawed in warm water (35 °C to 40 °C), washed and cultured on MS medium containing 2,4-D (0.2 mg l^{-1}), NAA (0.5 mg l^{-1}) and kinetin (0.1 mg l^{-1}). The retrieved embryos showed a lag period of up to 4 months without any sign of growth. In a few of these cultures, the embryos subsequently showed an overall swelling and elongation. The survival of a single coconut embryo, 15 months after freezing, was reported by Chin et al. (1989) using a classical protocol, i.e. cryoprotection with DMSO and slow freezing. Hornung et al. (2001) followed an encapsulation-dehydration protocol for the cryopreservation of plumular tissues of coconut in which the encapsulated plumular tissues were pre-cultured for 72–96 h in medium with 0.75 M sucrose and then desiccated with silica gel to around 30% moisture content. Callus growth was observed from the plumular tissues after freezing in liquid nitrogen.

Assy-Bah and Engelmann (1992a) could successfully establish rooted plantlets from coconut embryos from the coconut hybrid PB 121, frozen in liquid nitrogen. Immature embryos of coconut (7–8 months after pollination) were placed for 4 h in petri dishes on standard medium containing 600 g l^{-1} glucose. Pregrowth on this medium was compared with pregrowth on medium supplemented with the cryoprotectants glycerol, sorbitol or polyethylene glycol (PEG) 6000 at 5, 10 or 15%. Thereafter the embryos were immersed rapidly in liquid nitrogen. Thawing was carried out by immersion of the cryotubes for 30 seconds in a water bath at 40 °C. After freezing in liquid nitrogen, survival was obtained in three conditions only: pregrowth with 10% and 15% glycerol (25% and 10% survival, respectively) and 10% sorbitol (43% survival). PEG showed no cryoprotective effect at the concentrations tried. However, only one rooted plantlet could be obtained from embryos pretreated with 15% glycerol after 2.5 months.

Assy-Bah and Engelmann (1992b) reported cryopreservation of mature embryos of four varieties of coconut (Hybrid PB 121, Cameroon Red Dwarf, Indian Tall and Rennell Island Tall). The embryos were pretreated in the laminar air current for 4 h and subsequently incubated in a medium containing 600 g l^{-1} glucose and 15% glycerol for 11–20 h. After rapid freezing and thawing, a recovery rate of 33% and 93% was observed depending on the variety. Cryopreservation of mature embryos of West Coast Tall variety of coconut after desiccation pretreatments has been

reported. Maximum retrieval of healthy plantlets was achieved from embryos subjected to 18 h silica gel or 24 h laminar airflow desiccation treatment. When the moisture content of the embryo was reduced to below 20%, irreversible damage of shoot meristem was noticed (Karun et al. 2005).

Cryopreservation of coconut plumular tissues (apical dome with three to four leaf primordia) extracted from mature zygotic embryos of Malayan Yellow Dwarf cultivar has also been attempted. The plumular tissues were first pre-cultured on standard medium with 0.12 M sucrose for 3 days and subsequently suspended in standard medium containing 3% (v/v) Na-alginate and 0.15 M sucrose for encapsulation. After beads were made in 0.1 M calcium chloride containing 0.15 M sucrose, which was subjected to sequential pretreatment for 2–3 days in standard medium containing various sucrose concentrations (0.5 M, 0.75 M, 1 M). Thereafter, the beads were dried for 6–24 h on sterile filter paper over 40 g silica gel in 125 ml airtight boxes. After freezing, regrowth of plumules was obtained for plumules dehydrated for 14 h (21%) and 16 h (20%). Pregrowth of encapsulated plumule beads in 1 M sucrose for 16 h resulted in 20% leafy shoot production from the cryopreserved samples. These observations were consistent with the histological data showing similarity with control cells without any treatments (Nan et al. 2008).

Sisunandar et al. (2010a) put forth a modified and improved cryopreservation protocol for a number of Indonesian coconut cultivars. The protocol is comprised of four steps, viz. rapid dehydration, rapid cooling, rapid warming and recovery in vitro and acclimatisation and soil supported growth. For rapid dehydration, the embryos were positioned in a glass jar fitted with a stainless steel mesh (1–2 mm) platform and an autoclavable fan placed below the platform and housed in a polycarbonate tube. Activated silica gel (680 g) was placed into the lower portion of the glass jar in two separate zones. Following this protocol, 20% (when cryopreserved 12 days after harvesting) and 40% (when cryopreserved at the time of harvest) of embryos cryopreserved could be returned to normal seedlings. Differential Scanning Calorimetry (DSC) studies revealed that this procedure induced a drop in embryo fresh weight to 19% and considerably reduced the amount of water remaining that could produce ice crystals (0.1%). Of the 20 cultivars tested, 16 were found to produce between 10% and 40% normal seedlings, while 4 cultivars generated between 0% and 10% normal seedlings after cryopreservation. In a subsequent study, Sisunandar et al. (2010b) carried out morphological, cytological and molecular studies in coconut plantlets recovered after cryopreservation. The embryos from four cultivars were subjected to rapid dehydration in a drying chamber containing activated silica gel for 8 h to decrease the moisture content from 78% to 80% to 19% to 20%. The plants recovered from cryopreservation showed no morphological variation in terms of shoot elongation rates, production of opened leaves and number and total length of primary roots. In karyotype analysis, there was no variation in chromosome number ($2n = 32$), type of chromosomes, the length of the long and short arms, the arm length ratio and centromeric index in all studied cultivars independently of cryopreservation as revealed by karyotype analysis. Genetic and epigenetic fidelity of coconut plants recovered from cryopreservation was assessed through microsatellite (SSR) analysis and global DNA methylation rates; these

studies revealed that there were no significant differences between seedlings originating from cryopreserved embryos and respective controls.

Bandupriya et al. (2007) studied the effect of abscisic acid (ABA) on encapsulation-dehydration procedure for cryopreservation of coconut plumules. Supplementation of ABA (40 μM) to the sucrose pretreatment medium contributed significantly to the survival and recovery rates of cryopreserved plumules with 84% survival and 39% recovery. In a subsequent study, Bandupriya et al. (2010) investigated the most appropriate technique to transport and store mature zygotic embryos of coconut (for excision of plumules) for cryopreservation studies. The conditions tested include transportation as solid endosperm cores containing embryos (refrigerated for 10 days) and embryos in solidified agar or KCl solution (stored at 27 °C in dark for 10 days). Subsequent to encapsulation-dehydration, the plumule excised from embryos stored in KCl and solidified agar showed significantly higher rate of recovery than embryos in albumen cores in unfrozen samples. In frozen plumular tissues, there was no noteworthy difference in recovery under the conditions experimented. In frozen plumules pretreated with 1.0 M sucrose, the rate of recovery was significantly higher (40%) in the ones excised from embryos and stored in solidified agar in comparison to other conditions.

A protocol for cryopreservation of mature coconut embryos through vitrification has been developed by Sajini et al. (2011) after detailed studies on the effect of preculture conditions, vitrification and unloading solutions on survival and regeneration of coconut zygotic embryos after cryopreservation. PVS 3 solution, consisting of equal proportion of glycerol and sucrose, was most effective for regeneration of cryopreserved embryos among the seven plant vitrification solutions tested. The most efficient procedure included pre-culture of embryos for 3 days on medium with 0.6 M sucrose, followed by PVS 3 treatment for 16 h and subsequently cooling rapidly in liquid nitrogen and rewarming and unloading in 1.2 M sucrose liquid medium for 1.5 h. With this protocol, survival rates of 70% to 80% (corresponding to size enlargement and weight gain) and recovery of 20% to 25% (showing normal shoot and root growth) could be obtained from cryopreserved embryos. Sisunandar et al. (2014) examined the effect of maturity on the outcome of cryopreservation in four cultivars, viz. 'Nias Yellow Dwarf', 'Tebing Tinggi Dwarf', 'Takome Tall' and 'Bali Tall'. About 28% of plantlet recovery was achieved with 11-month-old nuts, which was considerably higher compared to younger nuts.

6.2.2.2 Pollen Cryopreservation

As viability of coconut pollen is only a few days (Patel 1938), alternative methods such as freeze drying was explored for short-term storage (Rognon and Nucé de Lamothe 1978; Whitehead 1963). Based on the results obtained by Karun et al. (2005) and Karun and Sajini (2010), it was concluded that storage of coconut pollen in liquid nitrogen for 24 h did not influence in vitro germination. Karun et al. (2014) have described procedures for long-term cryopreservation of coconut pollen of West Coast Tall and Chowghat Orange Dwarf cultivars. Pollen of both cultivars retained

their viability and fertility even after a storage period of 4 years in liquid nitrogen and gave normal nut set when used for pollination. One hundred per cent germination was observed in embryos extracted from hybrid nuts produced with cryostored pollen, and plantlet development was normal confirming the feasibility of setting up pollen cryobank in coconut.

Although field gene bank is the preferred means of conservation, embryo and pollen cryopreservation can also be taken up as complementary conservation strategies in coconut. Even when valuable genetic materials conserved in field gene banks are irrevocably lost, there is always a chance for their retrieval from the cryopreserved germplasm.

6.2.3 Tissue Culture

Mass multiplication of elite coconut palms, endowed with desirable traits like high yield, resistance to biotic and abiotic stresses, is of paramount importance in coconut. Research on coconut tissue culture started in the 1980s after success was reported in oil palm tissue culture. It was initially presumed that application of these techniques would be successful in coconut also. But the culture media developed for oil palm was indubitable for coconut, and it was later proved that the coconut palm is highly recalcitrant to in vitro manipulations and every stage of the procedure brought its share of problems (Verdeil et al. 1998). Poor in vitro responses, irrespective of explants, cultivars or culture conditions, have been attributed to the cumulative effect of several factors such as influence of genotype and explant maturity, adsorption of nutrients and hormones by activated charcoal making culture conditions undefined, production of compact calli, poor plantlet regeneration, underperformance of regenerated plantlets and very slow rate of growth during in vitro culturing (Fernando et al. 2010). Optimisation of composition of culture media, age and type of explants, plant growth regulators and their concentrations, period of subculturing and alternate additives, therefore, are contemplated for standardising a repeatable tissue culture protocol.

Selection of explants is a crucial factor in the success of in vitro experiments. After Eeuwens (1976) initial standardisation of media (Y3) and successful report of callus induction from various explant sources like stem, leaf and inflorescence, a few laboratories around the world initiated intensive research in this area. With respect to coconut, one of the foremost impediments is the lack of formation of friable embryogenic calli and formation of abnormal tissues. Many tissues, viz. leaves, inflorescence, ovary, anthers and zygotic embryos, have been utilised in tissue culture experiments. Coconut leaf explants from juvenile palms were made use of in some initial studies, and callus induction could be achieved (Pannetier and Buffard-Morel 1982; Raju et al. 1984). However, since the embryogenic capacity of leaf explants lasts only for a short duration, its use as an explant is limited (Karunaratne et al. 1991).

Immature inflorescence explants contain numerous meristematic points and therefore are a potential source in coconut, the important criteria being selection of inflorescence of correct maturity stage. Branton and Blake (1984), utilising immature inflorescence explants, observed that sections of inflorescence rachillae proliferated in medium to form calli, which they termed 'colloids'. Verdeil et al. (1994) reported formation of somatic embryos possessing functional bipolar organisation and completely differentiated shoot meristems from immature inflorescence explants. Vidhanaarchchi and Weerakoon (1997) reported that basal media composition influenced the responses of immature inflorescence explants, apart from its size. Raju (2006) reported that prolonged incubation of immature inflorescence explants in auxin-cytokinin combination media resulted in conversion of floral primordia to vegetative primordia, albeit at a very low frequency.

The earliest reports of using an in vitro anther culture approach in coconut are by Iyer (1981), Thanh Tuyen and De Guzman (1983) and Monfort (1985). Iyer (1981) reported formation of multicellular embryoids from coconut anthers. Stages of coconut inflorescence at which anthers and microspore are to be sampled were standardised and defined by Perera (2003). Perera et al. (2007, 2008) have reported formation of somatic embryo-like structures, possessing root and shoot apices, via anther culture. Success has been achieved in anther culture in a culture medium developed by Karunaratne and Periyapperuma (1989) supplemented with 9% sucrose (Perera et al. 2008, 2009). Addition of 0.1% activated charcoal was indispensable to lessen callus necrosis. Callus induction from microspores could be achieved in dark conditions in a medium supplemented with 2,4-D (100 μM), TDZ (9 μM) and NAA (100 μM). Maturation of the somatic embryos could be accomplished in a medium supplemented with 5 μM ABA and 10 μM $AgNO_3$ (Perera et al. 2007). Transfer of callus to a hormone-free medium and then to a medium supplemented with 5 μM BAP promoted germination of somatic embryos. Supplementations of 0.35 μM gibberellic acid along with 5 μM BAP further enhanced the germination frequency of mature somatic embryos (Perera et al. 2008, 2009). Ploidy analysis of anther-derived plantlets indicated that half of the regenerated plantlets were haploid and rest was diploid (Perera et al. 2008).

Unfertilised ovary has been reported as a promising source of explant with high regeneration potential – efficient callogenesis has been reported in CRI72 medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (Perera et al. 2007). Perera et al. (2009) reported 76% callusing frequency and 70% regeneration frequency from Sri Lankan Tall palms. A major advantage is that unfertilised ovaries can be extracted with minimal injury to the palm. Immature embryos of coconut (6–7 months post anthesis) cultured in medium supplemented with 2,4-D produced callus with 50% of them turning in to globular embryos. Around 22% of germination was observed in these cultures. Age of the embryo was considered as an important factor determining callus proliferation and subsequent embryogenesis (Karunaratne and Periyapperuma 1989).

The focus of a majority of recent studies has been the use of plumular tissues as explants, with promising and consistent results (Chan et al. 1998; Lopez-Villalobos 2002; Fernando et al. 2003; Saenz et al. 2006; Pe'rez- Nu'n˜ez et al. 2006; Rajesh

et al. 2005, 2014b). Multiplication of the embryogenic calli is the key to scaling up protocols; Perez-Nunez et al. (2006) could achieve rapid multiplication of embryogenic calli by subdividing and repeated subculturing of calli. Rajesh et al. (2005) described a regeneration protocol from plumular explants of West Coast Tall cultivar. Induction of callus was achieved in Y3 media supplemented with either an auxin alone (2,4-D; 74.6 μM) or in combination with a cytokinin (TDZ; 4.54 μM). Subculturing of calli at monthly intervals to media containing lower levels of 2,4-D and a constant level of either cytokinins (BA and TDZ) or polyamines (spermine and putrescine) resulted in formation of somatic embryos. Enhanced frequency of embryogenic calli, somatic embryoids and meristemoids were obtained in Y3 media supplemented with either spermine or BA. Evidences for development of shoot buds (organogenesis) and typical bipolar embryoids (somatic embryogenesis) were provided through histological studies. Albeit plantlets have been regenerated and successfully established in the field, a commercial scale protocol has not been accomplished, conversion of somatic embryos into plantlets remaining a major bottleneck. Various efforts have been made in coconut tissue culture to refine the protocol such as use of polyamines by Rajesh et al. (2014b) working with dwarf cultivars. Besides multiplication, maintenance of embryogenic callus for lengthy periods could ensure year-round supply of embryogenic calli. Bhavyashree et al. (2015) reported that coconut embryogenic calli obtained from plumular tissues could be maintained for 21 weeks without compromising on the embryogenic potential by subculturing the calli from lower 2,4-D concentration (74.6 μM/l) to higher levels (90.4 μM/l).

One of the major problems encountered during coconut tissue culture is browning, brought about by the release of secondary plant products especially polyphenols. Activated charcoal has been extensively used to avert browning (Samosir 1999). The supplementation of activated charcoal in the culture medium, however, hinders the action of the exogenously applied plant growth regulators and other media supplements, leading to uncertainties in the exact medium composition (Pan and van Staden 1998). The incidence of callogenesis and formation of somatic embryos have been reported to be influenced by variations in particle size and the potency of the different types of activated charcoal (Samosir 1999).

6.3 Molecular Studies

Knowledge of the extent of genetic diversity of a crop is a key element in any breeding programme. Traditionally, coconut genotypes have been characterised using morphological traits (Balakrishnan and Nair 1979; Panda 1982; Sugimura et al. 1997; Zizumbo-Villarreal and Piñero 1998). Assessment of genetic diversity using morphological markers has been difficult due to dearth of phenotypic markers, long juvenile phase of the crop and requirement of large-scale field trials, which is expensive. Molecular markers have helped to surmount these limitations as they are abundant, highly polymorphic and independent of environmental influences, and the

characterisation can be performed rapidly (Perera et al. 1998). In coconut, effective use of molecular markers has helped in characterisation and management of germplasm, estimation of extent of genetic diversity and population structure, linkage mapping, identification of QTLs for marker-assisted selection (MAS) and association analysis. Molecular markers used in coconut include biochemical markers (proteins, isozymes and polyphenols) and DNA-based markers [restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inverse sequence-tagged repeat (ISTR), simple sequence repeats (SSR), inter simple sequence repeats (ISSR), start codon targeted polymorphism (SCoT) and single-nucleotide polymorphism (SNPs)]. The use of these molecular methods has opened up new avenues for phylogenetic analysis and provided new tools for the efficient conservation and utilisation of coconut genetic resources.

6.3.1 Biochemical Markers

Initial studies on characterisation of genetic diversity in coconut involved proteins, isozymes, carotenoid or polyphenol markers (Benoit 1979; Jayasekera 1979; Carpio 1982; Jay et al. 1989; Fernando 1995). Work on isozyme analysis was first initiated in IRHO in 1978, with initial studies undertaken with pollen with nine enzyme systems (Benoit and Ghesquiere 1984). Four systems were selected and used to compare eight ecotypes; however, they demonstrated only a weak enzyme polymorphism, few polymorphic loci per system and not more than two alleles per locus. White et al. (1987) analysed six tall populations from Papua New Guinea and Solomon Islands using 15 enzymatic systems; polymorphism could be obtained only in four enzymatic systems with low diversity. Meunier (1992) analysed 6 tall populations from different origins with 17 isozyme systems. Although this method initially uncovered a clear distinction between ecotypes from Southeast Asia, Africa and Pacific regions (Bourdeix et al. 1993), its use was eventually abandoned due to marked environmental effects, low genetic diversity and low polymorphism. Fernando and Gajanayake (1997) utilised horizontal starch gel electrophoresis to characterise Sri Lankan coconut germplasm using six enzyme systems. Only two polymorphic loci, viz. peroxidases and esterases, could be recognised, which however are systems which have been frequently reported to be influenced by environment. Electrophoretic patterns of leaf peroxidase, endopeptidase and proteins were analysed in four cultivars and two hybrids by Cardena et al. (1998). The results obtained were utilised in coconut cultivar identification, testing progeny legitimacy, pollen contamination and studying breeding systems. Zizumbo-Villarreal et al. (2002) estimated the diversity in 22 Mexican and imported coconut populations using 15 enzymatic systems and reported low polymorphism. The fixation indices indicated low total heterozygosity and low heterozygosity within populations suggesting endogamy and genetic drift and a high diversity among populations due to differentiation between Pacific and Gulf of Mexico coastal populations. Forty

coconut cultivars from different geographical regions and 6 hybrids were analysed using 11 isozyme systems by Parthasarathy et al. (2004). Genetic diversity studies with enzyme systems came up against technical problems, as numerous systems proved monomorphic or not very active.

Other groups working with proteins (White et al. 1987; Canto-Canche et al. 1992) found that leaf extracts were easily oxidised, giving low enzyme activities and inconsistent results. On the other hand, analysis of leaf polyphenol polymorphism, using high-performance liquid chromatography (HPLC), provided a picture of variability that matched geographical origins and a clear distinction between talls and dwarfs (Jay et al. 1989). Chempakam and Ratnambal (1993) reported significant differences in the levels of leaf polyphenols in 36 cultivars from 8 different geographical origins. But the sensitivity of the polyphenol banding patterns to ecological conditions limits its applications. Presently, the characterisation of genetic diversity in coconut germplasm at the DNA level (Ashburner 1999) has largely replaced these strategies.

6.3.2 DNA-Based Markers

6.3.2.1 Diversity Studies

Restriction Fragment Length Polymorphism (RFLP): Lebrun et al. (1998) utilised RFLP technique to examine the genetic diversity in 100 palms, representing 10 tall and 7 dwarf coconut populations, from diverse geographical locations. Twenty cDNA probes from oil palm, rice, maize and coconut and one cytoplasmic probe from wheat were hybridised on digested DNA using four restriction enzymes. Based on molecular polymorphism, two genetically distinct groups could be defined – one group comprising of ecotypes from the Far East and the South Pacific and the other group comprising of ecotypes from Indian sub-continent and West Africa. All the dwarfs (except Niu Leka) formed a highly homogenous group related to the first group of talls. The results were in tune with earlier reports on the historical dispersion of coconut. The RFLP studies revealed substantial diversity in coconut populations from the Pacific and Far East regions, which are considered as putative regions of origin of the coconut palm. Furthermore, tall ecotypes, as expected, exhibited higher polymorphism compared to dwarf ecotypes.

Randomly Amplified Polymorphic DNA (RAPD): Seventeen distinct South Pacific populations displayed a moderate level of genetic diversity when analysed using 14 RAPD primers (Ashburner et al. 1997). Variability within the populations was over 60%. The low interpopulation diversity among observed coconut populations in this study was attributed to genetic drift and a possible bottleneck in the history of the species. A few RAPD markers unique to specific populations were also identified in the same study. RAPD technique has also been utilised to access the genetic diversity of coconut populations from Sri Lanka, the Philippines and Brazil as reported by Everard et al. (1996), Rodriguez et al. (1997) and Wadt et al. (1999), respectively.

Genetic diversity of four dwarf populations from East Java was analysed using RAPD (Hayati et al. 2000). Variability of coconut population grown outside East Java was reported higher than that at East Java since those coconut populations were raised from seeds of open pollinated palms.

Genetic diversity of 15 Indian and 5 exotic accessions of coconut was tested using 8 polymorphic RAPD primers (Upadhyay et al. 2004). The results revealed higher heterozygosity, higher proportions of polymorphic bands and higher genetic diversity in tall accessions compared to dwarf accessions. Likewise, exotic accessions displayed higher variation in comparison to Indian accessions. Dwarf accessions, from geographically distant regions, were found to group together. Genetic diversity studies within dwarf populations with yellow fruits, viz. Malayan Yellow Dwarf (MYD), Kulashekaram Yellow Dwarf (KYD) and Andaman Yellow Dwarf (AYD), analysed using 16 highly polymorphic RAPD primers, indicated the presence of greater diversity within accessions (Ritto et al. 2008).

Amplified Fragment Length Polymorphism (AFLP): AFLP analysis of 42 indigenous Sri Lankan genotypes, carried out using 8 primer pairs, revealed more variation in tall varieties (*typica*), rather than intermediate (*aurantiaca*) and dwarf (*nana*) varieties. *Aurantiaca* group was more similar to *nana* group rather than the typica group. In addition, putative duplicate accessions were identified in the a*urantiaca* group (Perera et al. 1998). Teulat et al. (2000) used AFLP markers in combination with SSR markers to analyse genetic diversity of 31 palms from 14 coconut populations from different ecological regions.

Inverse Sequence-Tagged Repeat (ISTR): Utilising inverse sequence-tagged repeat (ISTR) approach (primers complementary to repetitive copia-like sequences in the coconut genome), Rohde et al. (1992) could amplify a large number of genetic loci with an abundance of polymorphisms occurring among a set of selected coconut genotypes representing different regions of the world. Duran et al. (1997) extended this technique to analyse East African Tall coconut populations. It was concluded that ISTR markers constitute robust tools for genotype identification, analysis of germplasm variability and breeding purposes in coconut. These studies also provided evidence for the existence of truncated, copia-like repetitive sequences in the coconut genome indicating that retro-elements may have played a putative role in the generation of genetic diversity in coconut.

Inter Simple Sequence Repeats (ISSR): Thirty-three coconut accessions, comprising of coconut accession from different geographical regions of the world, maintained at the International Gene Bank in India, were analysed using 19 ISSR primers (Manimekalai and Nagarajan 2006). A total of 199 ISSR markers were generated, out of which 154 were polymorphic. Least similarity was found between Nicobar Tall and Chowghat Orange Dwarf, both accessions from India. Coconut accessions from Southeast Asia, South Asia and South Pacific formed distinct clusters, in accordance with their origin and dispersal.

Simple Sequence Repeats (SSR): The use of polymorphic microsatellites has gained popularity as a powerful tool for assessment of genetic diversity in coconut populations because of their high information content and codominant nature. Rivera et al. (1999) isolated 38 informative SSR markers from an enriched genomic

library. A preliminary screening of 20 coconut samples from the Philippine Coconut Authority (PCA) gene bank indicated the capability of these SSRs to detect substantial polymorphism with an average of 5.2 alleles per microsatellite. Additional confirmation using 40 coconut samples using 8 of the SSRs revealed an average of 8 alleles per SSR. Dwarfs grouped separately from talls and showed less genetic diversity, commensurate with their autogamous breeding behaviour. Using a precloning enrichment procedure, Perera et al. (1999) isolated eight coconut microsatellites, which were used to study the levels and patterns of genetic diversity of Sri Lankan coconut populations. The results showed that the Sri Lankan Tall coconuts (*typica*) exhibit higher levels of diversity than the dwarfs (*nana)* and intermediates (a*urantiaca*) and the intermediate coconuts are more similar to dwarfs than talls. This was in agreement with the results obtained using AFLPs in the same set of genotypes in an earlier study (Perera et al. 1998). Perera et al. (2000) used 8 pairs of SSR primers to analyse the genetic diversity in 130 individuals of coconut comprising 75 tall and 55 dwarf individuals representing 94 ecotypes from different geographical regions. A phenetic tree based on genetic distance clustered individuals into five groups, each mainly composed of either talls or dwarfs. Thirty-three tall coconut populations from Sri Lanka were subjected to microsatellite assay with eight SSR primer pairs in order to study the levels and distribution of genetic variation for formulating future collection strategies and selecting parents for breeding programmes (Perera et al. 2001). A high level of genetic diversity was detected in all the populations. A coconut microsatellite kit was developed by the CIRAD in collaboration with the COGENT, and it consists of 14 microsatellite markers with sufficient discriminating power for practical identification of coconut cultivars (Baudouin and Lebrun 2002). Standard protocols, without the use of radioactive probes, as well as dedicated statistical software, GeneClass2, were developed which could be adapted to use in developing countries.

Merrow et al. (2003) utilised 15 simple sequence repeat (SSR) microsatellite DNA loci to analyse genetic variation within coconut germplasm collections maintained at two locations in South Florida, USA, representing 8 cultivars. Parentage analysis of 'Fiji Dwarf' cultivar was also carried out using these loci. The Red Malayan Dwarfs were found to be genetically distinct from green and yellow ones. Also, genetic identity of 'Red Spicata' was found to be closer to 'Fiji Dwarf'.

A detailed assessment of the extent of genetic diversity in 21 Indian and 24 exotic coconut accessions was undertaken employing 8 SSR primers (Devakumar et al. 2006). The microsatellite loci distinguished a total of 48 alleles, with a mean of 6 alleles per locus. Genetic diversity values were low for most of the dwarfs and high for the tall accessions, which is in accordance with their breeding behaviour. However, Kulasekharam Orange Dwarf, an indigenous dwarf, showed genetic diversity higher than that of many of the tall populations. Within population variation (58%) was found to be higher than among population variation (42%).

Microsatellite analysis of lethal yellowing disease tolerant genotypes (Vanuatu Tall and Sri Lankan Green Dwarf) and susceptible genotype (West African Tall) was carried out by Konan et al. (2007). A total of 58 alleles were detected by the 12 microsatellite loci utilised for the study. Genotypes of susceptible West African Tall

cultivar were found to be less genetically clustered to the genotypes of the two tolerant cultivars. The fingerprinting based on microsatellites aided in identification of suitable parents to be used in crossing programmes for developing a segregating mapping population for marker-assisted selection of lethal yellowing resistant genes. Maypan coconut populations, a hybrid of Malayan Yellow Dwarf (MYD), and Panama Tall, earlier considered highly resistant, were devastated by an outbreak of lethal yellowing disease in Jamaica. Lebrun et al. (2007) used 34 SSR markers to compare the MYD sampled from 4 locations in Jamaica along with a reference DNA of MYD collected from 5 different countries, viz. Ghana, Malaysia, the Philippines, Mexico and India, to ensure whether the affected planting material in Jamaica was genetically similar to the material earlier shown to be resistant to lethal yellowing disease. The results revealed more variation at 34 simple sequence repeat loci in MYD samples from Jamaica than from the rest of the world. About 16% of alleles in Jamaican MYD samples did not match with the typical MYD genotypes indicating that Jamaican MYD palms were only partially true to type and this heterogeneity might have had an undesirable effect on its degree of resistance to lethal yellowing disease.

Rajesh et al. (2008a) studied the extent of genetic diversity in 26 coconut accessions from the Andaman and Nicobar Islands, India, utilising 14 SSR markers. A total of 103 alleles were obtained with an average of 7.35 alleles per locus. The average observed and expected heterozygosities were 0.29 and 0.66, respectively. Mean fixation index (F_{ST}) of 0.49 indicated a high level of population differentiation. More number of rare alleles was observed in tall accessions from the Nicobar Islands. Rajesh et al. (2008b) estimated the pattern of diversity in 10 landraces from 3 coconut-growing communities of India using 14 SSR markers. A total of 90 alleles were recorded with an average of 6.42 alleles per locus. Heterozygosity was highest for the tall landraces compared to dwarf ones.

Sixteen SSR markers were used by Dasanayaka et al. (2009) to detect genetic relationships of 43 coconut accessions conserved ex situ in the field gene banks of the Coconut Research Institute of Sri Lanka. These markers could uncover, without any ambiguity, the genetic relationships of Sri Lankan coconut populations. As expected and also reported in numerous earlier studies, gene diversity and polymorphism information content were relatively higher in allogamous tall coconut populations studied than in autogamous dwarf populations. Genetic lineages unveiled, based on evolutionary mechanisms, revealed a narrow genetic base of coconut germplasm, with most of the diversity restricted to the tall populations.

SSR markers were used to characterise two ecotypes of coconut from Kerala, India, namely, Annur and Bedakam. Clustering analysis shows their distinct nature as compared to local West Coast Tall (WCT) populations. However Annur ecotype was comparatively closer to WCT (Rajesh et al. 2014c). Rajesh et al. (2014d) studied the genetic and phylogenetic relationships of coconut populations selecting Laccadive Ordinary Tall (LCT) and Laccadive Micro Tall (LMT) from Amini and Kadamat Islands from Lakshadweep, India, using 20 polymorphic SSR markers. The variation was observed to be large among the distinct types of these cultivars. For example, the elliptical type from Amini emerged as a distinct type and was

found to be related to round type from Amini. The pear-shaped nuts from both islands showed affinity and seem to have formed as a result of introgression between elliptical and round types. The round form of LMT from Kadamat Island was found to be distinct according to SSR studies. The extent of genetic diversity and the population structure of four distinct coconut accessions (giant, ordinary, micro and mini micro types) of Minicoy Island, India, were carried out by using 19 SSR markers by Jerard et al. (2017). A total of 70 alleles were detected with a mean of 3.68 alleles per locus. The study revealed Laccadive Mini Micro Tall to be a genetically distinct accession.

Diversity and genetic relationships among 2 tall Brazilian coconut accessions ('Praia do Forte' and 'Merpe') and 7 accessions introduced from different geographic regions of the world ('Rennell Island Tall', 'Polynesian Tall', 'West African Tall', 'Malaysian Tall', 'Vanuatu Tall', 'Rotuman Tall' and 'Tonga Tall'), maintained at International Coconut Genebank for Latin America and the Caribbean (ICG-LAC), Brazil, were studied employing 19 polymorphic microsatellite primers. The primers could detect between four and ten alleles per locus, with an average of 6.57. The analyses unveiled the genetic relationships between Brazilian and African accessions and among the Southeast Asian and the South Pacific accessions, confirming the common origin of the accessions (Loiola et al. 2016).

Using 14 SSR markers, Rasam et al. (2016) characterised 5 indigenous accessions ('Banawali', 'Gangabondam Green Dwarf', 'Pratap', 'Konkan Bhatye Coconut Hybrid-I' and 'East Coast Tall') from the Konkan region of Maharashtra, India. The levels of polymorphism detected using SSR markers among the five accessions ranged from 85.7% to 100%. Existence of genetic variation, both between and within samples of each of the five accessions, was observed.

Diagnostic SSR markers have also been identified for use in hybridity testing in coconut in cases where morphological assessment, based on leaf petiole colour, is cumbersome (Perera, 2010; Rajesh et al. 2012). With the advances made in next-generation sequencing (NGS), the cost of development of molecular markers from non-model organisms has come down drastically. A few recent studies have reported the development and validation of SSR markers from expressed sequence tags (EST-SSRs) in assessment of genetic diversity in coconut (Xiao et al. 2013; Preethi et al. 2014) and genetic purity of coconut hybrids (Preethi et al. 2016).

Start Codon Targeted Polymorphism (SCoT): This novel marker system was described first by Collard and Mackill (2009) and is based on detection of short conserved region flanking the ATG translation start codon in plant genes. Genetic diversity in 23 coconut accessions (10 talls and 13 dwarfs), representing different geographical regions, was tested using SCoT marker technique. Results indicated the potentiality of SCoT markers in detection of DNA polymorphism in coconut accessions (Rajesh et al. 2015b).

WRKY Loci: Mauro-Herrera et al. (2006) utilised sequences of WRKY transcription factors, comprising of single-nucleotide polymorphisms (SNPs) and one microsatellite repeat, to generate ten informative markers. Fifteen genotypes, corresponding to six coconut cultivars, were analysed using these markers, and a total of two to four alleles could be detected. As an extension of this study, the

population used by Meerow et al. (2003) for SSR analysis was analysed by 13 WRKY markers. In spite of the lesser number of alleles detected using WRKY markers (37 alleles) compared to SSR markers (67 alleles), clustering of coconut populations using data derived from both these markers was comparable (Mauro-Herrera et al. 2007). Meerow et al. (2009) carried out detailed analysis of DNA sequences of 7 WRKY loci across 72 samples of Arecaceae tribe Cocoseae subtribe Attaleinae. Based on the results obtained, genus *Syagrus* was recognised as sister to *Cocos*.

Pesik et al. (2017) sought to evaluate nucleotide sequence diversities of *WRKY* gene in a collection of Kopyor (a mutant where endosperm is detached from its shell and forms endosperm crumbs in the shell) coconut germplasm in Indonesia. SNP-specific primers were designed based on eight informative SNPs identified, and duplex PCR could be successfully utilised for differentiating Banten Tall, Jember Tall, Kalianda Tall, Pati Dwarf and Sumenep Tall Kopyor coconuts.

6.3.2.2 Non-linkage Association of Markers with Phenotypic Traits

Attempts were made to identify RAPD markers associated with resistance to lethal yellowing disease using three coconut populations, viz. susceptible West African Tall (WAT), resistant Malayan Yellow Dwarf (MYD) and a resistant population of Atlantic Tall (AT) palms (Cardena et al. 2003). A particular RAPD band was regarded as associated with LY resistance if its frequency were high in MYD and surviving AT and low in WAT. Based on this criteria, a total of 12 markers were identified, and their possible use in marker-assisted breeding (MAS) was suggested. Shalini et al. (2007) carried out a detailed study to identify markers associated with resistance to coconut mite (*Aceria guerreronis* Keifer). A group of mite-resistant and mite-susceptible accessions from India were genotyped with 32 SSRs and 7 RAPD markers. Nine SSR and four RAPD markers, associated with mite resistance, were identified. Based on stepwise multiple regression analysis of each marker, a combination of six SSR showed 100% association with mite infestation, while three RAPD markers accounted for 83.86% of mite-resistant genotypes. A combination of five markers (three SSRs and two RAPD) could explain 100% of the association with mite resistance. In order to identify markers associated with palm habit, RAPD technique was employed to differentiate tall, dwarf and dwarf x tall hybrids using a bulked DNA approach (Rajesh et al. 2014a). The RAPD primer OPBA3 could clearly differentiate both the tall and dwarf bulks. The utility of the primer was validated in individual tall and dwarf coconut palms representing different geographic regions. This RAPD primer was also used to screen the parents and validate hybrids of dwarf x tall crosses. Purity of the dwarf x tall hybrids was tested using RAPD-derived sequence-characterised amplified region (SCAR) marker (Rajesh et al. 2013a).

6.3.2.3 Linkage Mapping and QTL Identification

An important step in genetic analysis is to produce genetic linkage maps which represent the relative order of genetic markers and their relative genetic distances from one another, along each chromosome of an organism. Co-segregation of markers with QTLs identified for agronomic traits can offer prospects for marker-assisted selection in coconut breeding programmes. Four DNA markers, viz. AFLP, ISSR, ISTR and RAPD, were utilised to generate the first linkage map in coconut using 52 progenies of a cross between Malayan Yellow Dwarf x Laguna Tall. A total of 382 markers generated 16 linkage groups for each parent with a total map length of 2226 cM. Six QTLs for early germination were identified; these QTLs were correlated with early germination and yield and represent characters which are important in coconut breeding (Herran et al. 2000). Lebrun et al. (2001) made use of 227 AFLP and SSR markers to construct a linkage map, consisting of 16 linkage groups and total map length of 1971 cM, utilising progenies of a cross between Cameroon Red Dwarf and Rennell Island Tall. Nine QTLs were detected for yield characters, viz. number of bunches and number of nuts. Using the same mapping population, Baudouin et al. (2006) investigated genetic factors which control fruit components in coconut. Out of the 52 putative QTLs identified for the 11 traits studied, 34 got grouped in 6 small clusters, which were presumed to correspond to single pleiotropic genes. Interestingly, QTLs for fruit component weight, endosperm humidity and fruit production were found at different locations in the genome, which implies the requirement for selection of QTLs for individual traits for efficient marker-assisted selection for yield. Waxes have been implicated in plant defences to different stress. Riedel et al. (2009) mapped QTLs related to cuticular wax on a linkage map derived from a population of 94 progenies of a cross between East African Tall and Rennell Island Tall using AFLP and SSR markers and COS clones. A total of 704 markers were placed onto the integrated map resulting in a total map length of 2739 cM. A total of 46 QTLs, relating to cuticular wax composition, could be mapped onto the coconut linkage map.

6.3.2.4 Association Analysis

Geethanjali et al. (2017) estimated the genetic diversity and population structure of 79 genotypes, representing accessions from around the globe, utilising 48 SSR loci. The number of alleles ranged from two to seven among the genotypes, with a mean of 4.1 alleles per locus. The genotypes exhibited reasonably high amount of genetic diversity, which was strongly structured based on their place of origin. Based on hierarchical clustering, the genotypes were grouped into two major clusters, each with two subclusters, consistent with their geographic origins. The first cluster encompassed tall genotypes from Indo-Atlantic and South Asia regions, while the second cluster consisted of dwarf genotypes and a few tall genotypes from Indo-Pacific and Southeast Asia. The occurrence of distinct genetic structuring in the accessions, with two major populations (K = 2) and four subpopulations (K = 4),

was corroborated by model-based clustering by STRUCTURE analysis. Only a low proportion of SSR locus pair (2.4%) was found to be in linkage disequilibrium. Association analysis attempted in a subset of 44 genotypes uncovered a SSR locus, viz. CnCir73, present in chromosome 1, putatively linked with fruit yield component traits (fruit breadth, kernel weight, nut weight and copra content), which was consistent with the results obtained in an earlier study (Baudouin et al. 2006).

6.4 Genomics and Transcriptomics

6.4.1 Gene Discovery

6.4.1.1 Somatic Embryogenesis

Various studies have been undertaken to understand and decipher the molecular basis of recalcitrance exhibited by different tissues in vitro. One of the first such studies was carried out by Perez-Nunez et al. (2009), who obtained the complete coding sequence (2240 nucleotides) of *somatic embryogenesis receptor-like kinase* (*SERK*), working with plumular explants. In situ RT-PCR studies revealed the spatial expression of *CnSERK* in meristematic centres among the embryogenic structures of calli. Expression of *CnSERK* could be detected in embryogenic calli even before somatic embryo formation could be observed, whereas it could be barely detected in non-embryogenic calli. From the results of this study, the use of *CnSERK* expression as a potential marker of competence of somatic cells to form embryos in coconut tissues cultured in vitro was suggested.

Montero-Cortés et al. (2010) isolated a putative cyclin-dependent kinase (*CDKA*), linked to cell cycle control, and carried out its expression analysis during somatic embryogenesis from plumular explants. Expression of *CDKA* was reported to be enhanced during embryogenic callus formation phase and progressively decreased as somatic embryos developed. Also, it was suggested that *CDKA* could be utilised as a marker to ascertain the meristematic capability and embryogenic competence of in vitro cultured tissues. For obtaining clues on genes expressed during somatic embryogenesis in coconut from transcriptome data of embryogenic calli, Rajesh et al. (2016) carried out detailed qRT-PCR analyses of 14 identified genes in 6 developmental stages of regeneration from plumular explants. The results revealed differential expression pattern of the 14 transcripts among different developmental stages: enhanced expression of *CLAVATA1* (CLV) was observed during the initial callogenesis; *germin-like protein* (GLP), *glutathione S-transferase*, *PICKLE* (PKL), *WUSCHEL* (WUS) and *WRKY* were expressed more in somatic embryos, while expression of *SERK*, *mitogen-activated protein kinase* (MAPK), *APETALA2/ethylene-responsive factor*, *SAUR family protein*, *embryogenic cell protein* (ECP), *late embryogenesis-abundant protein* (LEA), *arabinogalactan protein* (AGP) and *AINTEGUMENTA* (ANT) was higher in the embryogenic calli in comparison to initial callogenesis and somatic embryos. Bhavyashree et al. (2015)

attempted to correlate embryogenic potential of long-term maintained calli through gene expression studies. While enhanced expression of *ECP*, *GST*, *LEAFY* and *WUS* was recorded in long-term embryogenic calli (21 weeks old), expression of genes such as *SERK*, *GLP*, *WRKY* and *PK*L was higher in initial embryogenic calli (21 days old). From the results obtained, it was concluded that coconut plumular calli could be maintained for extended periods without loss in its embryogenic potential. In a later study, Bhavyashree et al. (2016) also carried out comparative studies of gene expression patterns during different stages of in vitro regeneration in two coconut cultivars, viz. a tall (WCT) and a dwarf (COD). The results of the study uncovered noteworthy differences in both the regeneration potential (higher somatic embryogenesis was recorded in WCT as compared to COD) and gene expression patterns (enhanced expression of *SERK*, *PKL* and *WUS* in embryogenic calli and GLP and *GST in* somatic embryos of WCT compared to COD), signifying genotypic differences of cultivars to in vitro regeneration.

6.4.1.2 Transcription Factors

An AINTEGUMENTA-like gene from coconut (*CnANT*), encoding two APETALA2 (AP2) domains and a conserved linker region, was cloned and characterised from a Sri Lankan Tall cultivar by Bandupriya et al. (2013). Comparison of genomic sequence of CnANT in tall and dwarf cultivars uncovered the presence of one single-nucleotide polymorphism and one indel in the first exon and first intron, respectively. A SSR marker, designed from within the indel sequence, could distinguish the tall and dwarf cultivars. Expression analysis by qRT-PCR indicated higher expression of CnANT in in vitro grown tissues in comparison to vegetative tissues. As an extension of this study, Bandupriya et al. (2014) reported enhanced expression of *CnANT* in mature zygotic embryos and also in embryogenic callus in comparison to other phases of somatic embryogenesis. Overexpression of *CnANT* in *Arabidopsis* resulted in induction of hormone-free regeneration of explants. Moreover, ectopic expression of CnANT improved in vitro regeneration, implying the role of *CnANT* in cellular proliferation in the course of in vitro culture. Sun et al. (2017) carried out detailed characterisation of Wrinkled (CoWRI1), an AP2/EREBP domain-containing transcription factor, which is an important regulator of oil accumulation, from coconut endosperm tissues. Transcriptional activities CoWRI1 and its interaction with acetyl-CoA carboxylase promoter were established by the yeast two-hybrid and yeast one-hybrid approaches, respectively. Ectopic expression of *CoWRI1*, undertaken through seed-specific expression in *Arabidopsis* and endosperm-specific expression in rice, appreciably enhanced oil content in the seeds of transgenic *Arabidopsis* and *rice*.

6.4.1.3 Endosperm Development

Knutzon et al. (1995) cloned the coding region of lysophosphatidyl acyltransferase (LPAAT), a pivotal enzyme controlling the metabolic flow of lysophosphatidic acid into different phosphatidic acids, from a coconut endosperm cDNA library. The upstream region of *LPAAT* from coconut genome was amplified employing chromosome walking, and a number of putative promoter elements were described, including TATA-box, CAAT-box and Skn1-motif. The expression pattern of coconut *LPAAT* promoter, based on stable genetic transformation in transgenic rice plantlets, revealed that the expression was confined to rice endosperm (Xu et al. 2010).

Li et al. (2009) compared the expression profiles of miRNAs at two developmental stages in coconut endosperm development [immature (8 months) and mature (12 months)], using miRNA microarray assays, to categorise miRNAs involved in endosperm tissue development and compound anabolism. A total of 179 miRNAs were recorded in immature (95 expressed miRNAs) and mature tissues (176 expressed miRNAs), based on the annotation in miRBase. A total of 32 miRNAs displayed differential expression (23 were upregulated and 9 downregulated in mature endosperm), thereby associating the role of miRNAs in development of coconut endosperm. The differential expression patterns of a few of these miRNAs were validated using qRT-PCR. Also, the target genes of 32 miRNAs, with differential expression patterns, were computationally predicted. Liang et al. (2014), utilising suppression subtractive hybridisation, identified 737 unigenes which were differentially expressed during 3 developmental stages of coconut endosperm. Out of these, 103 were found to encode enzymes implicated in fatty acid and carbohydrate biosynthesis and metabolism. Stage-specific expression of selected transcription factors and other relevant genes were established by qRT-PCR.

6.4.1.4 Resistant Gene Analogues

Transcriptome data of leaf samples of coconut root (wilt) disease-resistant cultivar (Chowghat Green Dwarf) were mined, and 243 resistance gene analogue (RGA) sequences were identified, encompassing 6 classes of RGAs, by detailed domain and conserved motif predictions by computational analysis (Rajesh et al. 2013b, 2015a, 2017). Phylogenetic analyses grouped coconut nucleotide binding site and leucine-rich repeat (NBS-LRR) class of *R* genes into discrete clusters, based on the occurrence of either TIR or CC motifs in the N-terminal regions. In addition, the expression pattern of a few NBS-LRR type RGAs was validated through qRT-PCR analysis. Using degenerate primers, Puch-Hau et al. (2015) amplified NBS-type RGAs from coconut accessions displaying either resistance or susceptibility to the lethal yellowing disease. Based on phylogenetic analysis of the sequences obtained, seven distinct clades were obtained, with all sequences belonging to the non-TIR-NBS-LRR subclass of NBS-LRR type RGAs. Variations in expression profiles of a few of these transcripts were observed in response to exogenous application of

salicylic acid. In a later study, Puch-Hau et al. (2016) reported species-specific evolution of resistance gene candidates in coconut palm.

Utilising primer pairs based on conserved motifs of the NBS-LRR domain of the date palm, Rachana et al. (2016) amplified putative RGAs from Chowghat Green Dwarf. The isolated RGAs were homologous to monocot NBS-LRRs. Detailed structural analysis and 3-D modelling of the NBS domain of these RGAs were also carried out.

6.4.1.5 Aroma

The presence of 2-acetyl-1-pyrroline (2AP) imparts a pleasant 'pandan-like' aroma to liquid endosperm of Aromatic Green Dwarf from Thailand. Saensuk et al. (2016) identified an ortholog of the rice aromatic gene in coconut (*CnAMADH2*). Comparison of coding sequences of *CnAMADH2* of Aromatic and non-Aromatic Green Dwarf palms uncovered a modification of guanine (G) in non-aromatic accession to cytosine (C) in the aromatic accession in the exon 14, which caused a non-synonymous amino acid change from alanine (A) to proline (P) at position 442 (P442A) of CnAMADH2. This substitution was linked to 2AP content in the aromatic palms. Based on this sequence variation, a PCR marker, capable of detecting aromatic and non-aromatic alleles of the gene, was developed and validated.

6.4.2 Estimation of Genome Size

Sandoval et al. (2003) carried out flow cytometer analysis of different tissues (immature leaves, shoot meristems extracted from zygotic embryos and culture in vitro and slow- and fast-growing calli) and estimated the average nuclear DNA content of coconut to be 5.6 ± 0.2 pg DNA/2C ($\simeq 5.4 \times 10^9$ bp). Utilising results obtained from flow cytometry studies of 23 coconut cultivars (comprising of talls, dwarfs and a hybrid), Gunn et al. (2015) estimated the average genome size to be around 5.96 pg, using isolated nuclei from young palm leaves. They concluded that the coconut genome is large and displayed intraspecific variation linked to domestication, with variations among talls significantly more in comparison to dwarf cultivars. In a later study, Neto et al. (2016) estimated the genome sizes of 14 coconut accessions, comprising of 8 tall and 6 dwarf cultivars, using flow cytometry. The 2C DNA content varied from 5.72 to 5.48 pg for talls and from 5.58 to 5.52 pg for dwarf accessions, and the mean genome size was estimated to be 5.59 and 5.55 pg for the tall and dwarf accessions, respectively.

6.4.3 Chloroplast Genome

Huang et al. (2013) described the chloroplast genome sequence of a dwarf coconut palm and provided detailed account of its gene content and organisation, inverted repeat fluctuations and repeated sequence structure, in addition to occurrence of RNA editing. Chloroplast genome of coconut, about 154,731 bp in length, was predicted to encode 130 genes and 4 pseudogenes. Albeit the smallest reported so far in palms, chloroplast genome of coconut shared the same overall organisation, gene content and repeat structures with chloroplast genome of other palms. Pseudogenisation of *rps19*-like gene and an unusual high number of RNA editing sites are a couple of unique features encountered in the coconut chloroplast genome.

6.4.4 Mitochondrial Genome

The assembly of complete coconut mitochondrial (mt) genome of an Oman Local Tall cultivar was reported by Aljohi et al. (2016). The mt genome is around 678.65 kbp in length, possessing a GC content of 45.5%, and was found to encode 72 proteins, 9 pseudogenes, 23 tRNAs and 3 ribosomal RNAs. The chloroplast (cp)-derived regions accounted for 5.07% of the total assembly length in contrast to the date palm mt genome, where 93.5% of the genome sequence was found to be cp derived (Fang et al. 2012). In coconut, the cp-derived regions included 13 proteins, 2 pseudogenes and 11 tRNAs. The mt genome of coconut has a relatively large fraction of repeat content (17.26%), including both forward (tandem) and inverted (palindromic) repeats. Sequence variation analysis shows that the transition/transversion ratio of 0.3 in the mt genome was much lower (2–2.1) when compared to that of the nuclear genome.

6.4.5 Transcriptome Analysis

6.4.5.1 Host–Pathogen Interactions

The first genome-wide study of coconut using transcriptome analysis was carried out by Rajesh et al. (2013b) to ascertain the intricate host-pathogen interactions with respect to root (wilt) disease in coconut. Reads obtained from RNA-Seq analysis of healthy and diseased Chowghat Green Dwarf (CGD) palms, utilising an Illumina HiSeq 2000 platform, were assembled into 59, 282 transcripts, with a mean size of 987 bp (Rajesh et al. 2017). Based on sequence similarity searches, 39,665 of assembled transcripts had at least 1 significant hit in the Uniprot and date palm proteome databases. Overall, 2718 transcripts were differentially expressed in diseased samples (fold change of 2 and above with a p value < = 0.05) in

comparison to healthy ones. The differentially expressed transcripts could be sorted out into pathways like formation of cell wall, plant-pathogen interactions, primary and secondary metabolism, biosynthesis of hormones and signalling cellular transport. The expression patterns of a set of genes, both upregulated and downregulated, were validated by quantitative real-time PCR (qRT-PCR), the results of which were comparable to those observed by RNA-Seq analysis.

Nejat et al. (2015) undertook RNA-Seq analysis to distinguish the global transcriptome responsive to coconut yellow decline disease by comparing the RNA-Seq profiles of naturally infected and healthy Malayan Red Dwarf palms. Illumina sequencing generated a total of 72,019,264 and 70,935,896 reads, which could be assembled into 108,994 and 148,264 contigs from the healthy and infected leaf transcriptomes, respectively. A total of 18,013 transcripts were upregulated, and 21,860 transcripts downregulated in infected leaves in comparison to healthy ones. The results obtained revealed reprogramming of many of the biological and cellular processes due to phytoplasma infection. A number of genes associated with the production of defence-related proteins, reactive oxygen species, ABC transport protein family, hydrolase and kinases, flavanol synthesis, auxin-induced protein, no apical meristem (NAM) gene family and ethylene were upregulated as a result of phytoplasma infection. Upregulation of *GA-2ox* (gibberellin 2-oxidase) was presumed to downregulate levels of gibberellins in infected coconut palms; this could be the possible reason for stunting, inflorescence necrosis and premature nut fall, which are the characteristics of phytoplasma infection in coconut. Genes involved in photosynthesis were downregulated and could constitute diversion of cellular machinery towards defence mechanisms in response to biotic stress. It was also implied that phytoplasma might modify the expression of genes involved in carbohydrate metabolism in infected palms. The upregulation of ABC protein family transcripts during phytoplasma infection could be construed as a mechanism by phytoplasma to import sugars, which are their main source of energy, through the ABC transporter systems.

6.4.5.2 Fatty Acid Biosynthesis and Metabolism

Fan et al. (2013) undertook transcriptome analysis combining RNA isolated from juvenile leaves and fruit flesh of Hainan Tall cultivar. A total of 57,304 unigenes could be obtained after assembly of the RNA-Seq data, with an average length of 752 bp. Out of these, 23,168 unigenes could be mapped into 215 KEGG pathways, mainly galactose metabolism, hormone signal transduction and plant-pathogen interaction pathways. Additionally, 347 unigenes involved in fatty acid synthesis and metabolism were also reported. These unigenes could be assigned to five steps of the fatty acid biosynthesis pathway (fatty acid biosynthesis, unsaturated fatty acid, citrate cycle, fatty acid metabolism and fatty acid elongation), thus aiding elucidation of the molecular mechanisms involved in fatty acid biosynthesis in coconut. Of these, 20 unigenes were predicted to be related to fatty acyl-ACP thioesterase, which is a key enzyme for terminating the elongation of carbon chains

and therefore regulating the length of fatty acids. From the leads obtained in this study, it was proposed that expression of fatty acyl-ACP thioesterase might be associated with the observed accumulation of medium chain fatty acids (i.e. lauric acid) in coconut.

6.4.5.3 Embryogenesis

De novo assembly and analysis of global transcriptome of coconut embryogenic calli, derived from plumular explants of West Coast Tall cultivar, was carried out using Illumina paired-end sequencing. From the assembled reads, transcripts known to be involved in SE, namely, protein kinases like receptor-like kinases (*SERK* and *CLV1*), mitogen-activated protein kinase (*MAPK*), transcription factors (*WUS*, *AP2/ERF*, *PKL*, *ANT* and *WRKY*), extracellular proteins (*AGP*, *GLP*, *ECP* and *LEA*) and *GST*, were mined (Rajesh et al. 2016). Bandupriya and Dunwell (2016) carried out a comprehensive study of ESTs by exploring the transcriptome data of immature embryo, mature embryo, microspore-derived embryo and mature leaves to categorise key embryo-specific genes. Transcripts with putative roles in embryogenesis, viz. chitinase, β-1,3-glucanase, ATP synthase CF0 subunit, thaumatin-like protein and metallothionein-like protein, were identified.

6.4.5.4 RNA-Directed DNA Methylation

Huang et al. (2014) carried out transcriptome analysis of maturing gelatinous endosperm, mature embryo and young leaf of a fragrant dwarf coconut. After assembly of the sequencing data, a total of 58,211, 61,152 and 33,446 unigenes could be identified from embryo, endosperm and leaf tissues, respectively. Putative homologues of factors required for RNA-directed DNA methylation in coconut were identified. The results imply the importance of RNA-directed DNA methylation, especially small RNA-mediated epigenetic regulation during seed development, particularly in maturing endosperm, in coconut.

6.4.5.5 Aroma

De novo assembly of transcriptome from the liquid endosperm of Aromatic Green Dwarf coconut of Thailand was undertaken by Saensuk et al. (2016) to identify the gene(s) responsible for biosynthesis of 2-acetyl-1-pyrroline (2AP) biosynthesis, which imparts a ‘pandan-like’ aroma. From the assembled whole-transcriptome data, derived through RNA-Seq of 7-month-old endosperms of Aromatic and a non-Aromatic Green Dwarf coconut, size differences were observed in transcripts encoding 2AP in aromatic (2371 bp) and non-aromatic (1921 bp) palms. These transcripts were orthologous to rice 2AP.

6.4.5.6 Whole Genome Sequencing

Recently, the whole genome sequence of coconut (cultivar Hainan Tall) was made available, and a total of 419.08 gigabases (Gb) of clean data was obtained (Xiao et al. 2017). This study generated a scaffold length of 2.2 Gb (with a scaffold N50 of 418 kb) that represents over 91% of the calculated genome of coconut. The study further predicted that coconut genome harbours 28,039 protein-coding genes compared to *Phoenix dactylifera* (PDK30 variety: 28,889), *Phoenix dactylifera* (DPV01 variety: 41,660) and *Elaeis guineensis* (34,802). Around 72.75% of the genome comprise transposable elements with long-terminal repeat elements (LTRs) contributing to a little over 92% of transposable elements in coconut (Xiao et al. 2017). In addition, the completeness of genome, analysed using BUSCO, showed that 90.8% of the 1440 expected plant genes were identified as complete. Bayesian molecular clock analysis showed that the divergence time between coconut and oil palm is about 46.0 (25.4–83.3) million years ago. Comparative analysis of coconut and *Arabidopsis thaliana* encoded antiporter and ion channels revealed that coconut has acquired significant gene family expansions including Na+/H+ antiporters, carnitine/acylcarnitine translocases, potassium-dependent sodium-calcium exchangers and potassium channels. Further, it was also inferred that the expansion of these gene families could be ascribed to the adaptation of coconut to salt stress, fatty acids and potassium accumulation.

6.5 Transformation Studies

Samosir (1999) attempted genetic transformation studies for the first time in coconut utilising microprojectile bombardment for targeting *GUS* gene into embryogenic calli and juvenile leaf tissues. It was reported that two constitutively expressed promoters, viz. actin (*Act1*) and ubiquitin (*Ubi*), produced the strongest transient expression in these tissues. Andrade-Torres et al. (2011) described *Agrobacterium*-mediated transformation of a wide range of tissues such as immature anthers, excised zygotic embryos, plumule-derived embryogenic calli and somatic embryogenesis-derived roots and leaves. A number of reporter genes and procedures for antibiotic selection of transformants were evaluated. Since embryogenic calli displayed endogenous *GUS*-like activity, other genes (e.g. green/red fluorescent protein) were experimented with. From the results obtained, it was concluded that successful gene transfer could be achieved by a combination biolistics (to generate micro-wounds in explants) and *Agrobacterium*-mediated transformation (to introduce genes into explants).

6.6 Future Strategy

Despite its importance as a crop in the tropics, the genetics of coconut has not received much attention in comparison with many other crops. The genetic basis of inheritance of important traits has to be understood. Coconut remains a difficult crop to manipulate in vitro. A viable protocol for micropropagation of desired coconut hybrids/selections has to be developed to enable disseminating the benefits of various breeding programmes. The technique, when perfected, could also be used for the mass multiplication of the disease-resistant/tolerant types especially, in the context of the epidemic and devastating nature of lethal yellowing and root (wilt) diseases. Embryo culture, embryo rescue and cryopreservation techniques have helped in collection and preservation of germplasm. The important advances in biotechnological techniques which have been made particularly during the last decade, such as identification of molecular markers associated with polymorphism and potentially linked to specific traits, should be streamlined to benefit the coconut breeder in practice.

The use of new-generation sequencing techniques has witnessed large-scale generation of ESTs in coconut, which has provided insights into plant-pathogen interactions and facilitated elucidation of genes involved in somatic embryogenesis. The available genome sequence forms a potential resource to be utilised in genetic enhancement of coconut palm. It is anticipated that a large volume of genomic resources, with respect to transcriptome sequencing and whole genome sequencing using next-generation sequencing platforms, would be made available soon in the public domain due to the initiatives undertaken by numerous laboratories around the globe. The obvious challenge to unravel and integrate the genomic knowledge into appropriate methodologies should be taken up which would revolutionise future coconut breeding programmes, especially in terms of palm productivity and resistance to biotic and abiotic stresses, and lead to deciphering in vitro recalcitrance.

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