

DNA polymorphism among coconut (*Cocos nucifera* L.) cultivars and reciprocal cross derivatives differing in drought tolerance

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Abstract

Coconut seedlings of Laccadive Ordinary Tall (LCT), Gangabondam Green Dwarf (GBGD) and their reciprocal cross combinations were analysed by molecular markers viz. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR). These seedlings were evaluated for water stress tolerance based on leaf water potential measured during stress and non-stress periods. The leaf water potential decreased during stress period. The differences among the individuals for leaf water potential during stress were not significant. The molecular markers revealed the genetic structure of the cultivars and derivatives clearly. In the dendrogram, individuals of GBGD and GBGD X LCT formed one cluster and LCT and LCT X GBGD formed another cluster. The individuals of LCT and LCT X GBGD showed variation among themselves and they were scattered in the principal co-ordinate plot. Even though the parents are same, the reciprocal cross derivatives behaved differently for leaf water potential and showed polymorphism at DNA level. When the dwarf palm was used as female parent, the F_1 s were similar to female parent in their banding pattern.

Key words : Coconut, DNA, RAPD, ISSR, drought tolerance

Introduction

The ability of coconut genotypes to withstand drought conditions depends upon various physiological and biochemical factors, which impart drought tolerance. Unlike annuals, the adverse effect of drought persists for the subsequent two to three years and this is explained on the basis of the relationship between degree and intensity of dry spell and the ontogeny of coconut inflorescence (Rajagopal *et al.*, 1991).

The basic physiological principle in drought studies is to look for the genotype which can conserve water and maintain leaf turgidity during adverse conditions. This can be assessed through the determination of leaf diffusive (stomatal) resistance, reflecting the degree of transpirational rate and leaf water potential or the relative water content. Based on this principle, Rajagopal *et al.* (1990) screened 23 coconut genotypes both during stress and non-stress periods. Their result revealed that genotypes like West Coast Tall inter-

se (WCT X WCT), Federated Malay States, Java Giant, Andaman Giant, LCT X GBGD, LCT X COD (Chowghat Orange Dwarf), WCT X COD and WCT had higher stomatal resistance with low rate of transpiration.

Rajagopal *et al.* (1990) measured leaf water potential (LWP) using Scholander pressure chamber technique. During stress period the LWP ranged from -1.19 Mpa to -1.57 Mpa among the genotypes. Kasturi Bai *et al.* (2001) measured the variation in leaf water potential and peroxidation among the coconut seedlings of West Coast Tall (WCT), Laccadive Ordinary Tall (LCT), Philippines Ordinary Tall (PHOT), Andaman Ordinary Tall, (ADOT), Chowghat Green Dwarf (CGD), and the reciprocal cross derivatives (T X D's and D X T's). Higher water potential was observed in LCT, CGD X PHOT, CGD X ADOT during stress period.

Among the different types of molecular markers, Random Amplified Polymorphic DNA (RAPD) is fast and these markers are easily generated by PCR (Williams

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et al., 1990). In RAPD banding profiles are created using small oligo nucleotide primers (around 10 bp in length) of arbitrary sequence.

Inter-simple sequence repeats (ISSR) technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellite, usually 16-25bp long, as primer in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. The microsatellite repeats used as primer can be a di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide. The technique combines most of the benefits of AFLP and microsatellite analysis with the universality of RAPD. ISSR's have high reproducibility possibly due to the use of longer primers (16-25-mers) as compared to RAPD primers (10-mers), which permits the use of high annealing temperature (45-60°C) leading to higher stringency.

ISSR markers are highly polymorphic and are used in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology. ISSR-PCR is a technique which overcomes the problems like low reproducibility of RAPD, high cost of AFLP, the need to know the flanking sequences to develop species-specific primers for SSR polymorphism (Godwin *et al.*, 1997). ISSRs segregate mostly as dominant markers following simple Mendelian Inheritance (Wang *et al.*, 1998). However, they have also been shown to segregate as co-dominant markers in some cases, thus enabling a distinction between homozygote and heterozygote (Sankar and Moore, 2001).

Fingerprinting accessions differing in drought tolerance had been done earlier in rice (Linu *et al.*, 2000). So far, there are no reports in coconut regarding molecular characterization for drought tolerance or fingerprinting. There is a report on identification of molecular markers for lethal yellowing (Cardena *et al.*, 2003).

In this paper, molecular markers *viz.* RAPD and ISSR were employed to reveal the genetic structure and relationship among a set of coconut cultivars and derivatives to establish the find presence of similarities if any with the leaf water potential pattern. Leaf water potential has been extensively used in screening for drought tolerance in crop plants, and in coconut a method for rapid screening for drought tolerance has been developed based on this trait (Rajagopal *et al.*, 1988).

Materials and Methods

Leaf water potential

Twelve-month-old seedlings belonging to two cultivars, LCT and GBGD, and their reciprocal crosses (Table 1) growing under pot culture conditions were used. The sample size consisted of 3-4 seedlings. The seedlings were watered daily and exposed to water stress. Leaf water potential (bars) was measured following the method of Scholander *et al.* (1964). From each seedling, the leaflet from the third leaf was excised and immediately placed in pressure chamber to avoid transpirational water loss. Leaf water potential was determined between 10.00 to 11.30 AM.

Table 1. Leaf water potential (-bars) in coconut seedlings during non-stress and stress periods

Cultivar	Control	Stress	Difference	% Variation over pre stress
LCT1	10.30	13.80	3.50	34.0
LCT2	10.00	12.60	2.60	26.0
LCT3	6.88	14.40	7.52	109.3
LCT4	8.30	11.60	3.30	39.8
	8.87	13.10	4.23	52.3
LCT x GBGD1	6.92	17.40	10.48	151.4
LCT x GBGD2	10.00	13.00	3.00	30.0
LCT x GBGD3	12.00	15.90	3.90	32.5
	9.64	15.43	5.79	71.3
GBGD1	9.39	11.90	2.51	26.7
GBGD2	6.32	10.20	3.88	61.4
GBGD3	4.97	11.00	6.03	121.3
GBGD4	7.40	14.20	6.80	91.9
	7.02	11.83	4.81	75.3
GBGD x LCT1	7.30	13.30	6.00	82.2
GBGD x LCT2	6.50	16.50	10.00	153.8
GBGD x LCT3	5.40	14.23	8.83	163.5
GBGD x LCT4	8.00	14.35	6.35	79.4
	6.80	14.60	7.80	119.7

Table 2. Mean leaf water potential among cultivars and hybrids during stress and non stress

S.No	Cultivar/ hybrid (A)	Non stress	Stress (S)	A-Mean	Diff
1	LCT	8.8700 a	13.1000 a	10.9850 a	-4.2300 **
2	LCTXGBGD	8.5100 a	14.3250 a	11.4175 a	-5.8150 **
3	GBGD	7.0200 a	11.8250 a	9.4225 a	-4.8050 **
4	GBGDxLCT	6.8000 a	14.5950 a	10.6975 a	-7.7950 **
	S-MEAN	7.8000	13.4613	10.6306	-5.6613 **

** = significant at 1% level

RAPD analysis

DNA was extracted from 2 g of fresh leaf material following the protocol of Upadhyay *et al.* (1999). For RAPD analysis amplification reactions were carried out in 10l volume containing 30 ng of template DNA, 200 µM of each dNTPs, 0.36 U of Taq polymerase (Bangalore

Table 3. List of Operon primers and number of polymorphic bands.

S.No	Primer	Total bands	No. of polymorphic bands
1	OPAG 18	9	4
2	OPAL 11	9	8
3	OPAG 13	12	4
4	OPB 15	9	5
5	OPBE 01	6	1
6	OPBE 12	7	2
7	OPBE 13	6	4
8	OPBE 14	12	7
9	OPBE 15	5	2
10	OPBE 16	9	7
11	OPBE 18	8	4
12	OPBE 3	12	7
13	OPBE 2	17	12
14	OPBE 4	6	4
15	OPBE 5	14	12
16	OPBE 6	17	17
17	OPBE 7	9	3
18	OPBE 8	12	7
19	OPC 10	12	6
20	OPC 12	13	5
21	OPC 13	16	13
22	OPC 15	6	5
23	OPC 3	12	7
24	OPC 4	17	8
25	OPC 7	14	8
26	OPC 8	5	2
27	OPC 9	10	4
28	OPE 1	12	1
29	OPE 11	14	11
30	OPE 12	7	5
31	OPE 16	12	10
32	OPE 18	18	5
33	OPE 2	14	11
34	OPF 14	18	14
35	OPG 8	10	5
36	OPG 9	15	8
37	OPM 10	8	4
38	OPM 2	17	16
39	OPM 11	8	3
40	OPM 17	14	12
41	OPM 20	12	8
42	OPM 4	12	9
43	OPM 6	19	13
44	OPM 7	12	7
45	OPM 9	9	7
	Mean	11.44	7.04
	Total	515	317

Genei Pvt. Ltd. India) and 25 pmol of random primer (Operon technologies, Alameda, USA). Amplifications were performed in a Thermo Cycler (MJ Research Inc.) programmed for an initial denaturation at 94 °C for 2 min., 40 cycles of 1 min. denaturation at 94 °C, 1 min. annealing at 37 °C and 2 min. extension at 72 °C and a final extension of 7 min. at 72 °C. The PCR products were subjected to electrophoresis through a 1.80 % agarose gel using 1 X TBE buffer at 90 volts for 2 h in

Bio-Rad submarine electrophoresis unit. The ethidium bromide stained gels were documented using the Alpha Imager™ 1200 – Documentation and Analysis system of the Alpha Innotech Corporation, USA. Forty-five operon primers were used in the study (Table 3). The PCR was repeated for each primer with two DNA samples to monitor the reproducibility of banding pattern.

ISSR analysis

A total of nineteen primers were used for the study (Table 4). Amplification reactions were carried out as in RAPD and the thermal cycling was programmed for initial denaturation at 94 °C for 5 min., 40 cycles of 1 min. denaturation at 94°C, 1 min. annealing at 47°C, 2 min. extension at 72°C, final extension of 5 min. at 72°C and then at 4°C till storage.

Table 4. List of ISSR primers and number of polymorphic bands

S.No	Primer	Total number of bands	No. of polymorphic bands
1.	UBC 815	7	6
2.	UBC 823	9	5
3.	UBC 824	10	7
4.	UBC 827	5	4
5.	UBC 834	12	6
6.	UBC 835	8	5
7.	UBC 841	8	4
8.	UBC 842	9	5
9.	UBC 854	8	5
10.	UBC 855	10	7
11.	UBC 856	10	8
12.	UBC 807	12	7
13.	UBC 808	15	12
14.	UBC 814	5	3
15.	UBC 836	6	3
16.	UBC 864	5	0
17.	UBC 868	6	1
18.	UBC 889	12	11
19.	UBC 810	12	9
	Mean	8.90	7.2
	Total	169	108

Data analysis

Only the clear, unambiguous and reproducible bands were considered for scoring. Each band was considered to be a single locus. Data were scored as “1” for the presence and “0” for the absence of a DNA band of each accession.

The binary data matrices were entered into the NTSYS pc package (Exeter Software, New York). The data were analysed using Qualitative routine to generate Jaccard's similarity coefficient. Similarity coefficients were used to construct a dendrogram using UPGMA (Unweighted Pair Group Method with arithmetic Average) and the SHAN (Sequential Hierarchical and

nested clustering) routine in the NTSYS program. The principal coordinate analysis (PcoA) was conducted with NTSYS software.

Matrix comparison

Similarity matrix produced by RAPD and ISSR were compared by the MXCOMP routine of NTSYS pc. The normalized Mantel statistic Z (Mantel, 1967) was used to determine the level of association between the matrices.

Results and Discussion

Leaf water potential

Leaf water potential ranged from between - 4.97 bars (GBGD) to -12.0 bars (LCT X GBGD) among the individuals during unstressed conditions. During stress it ranged from -10.20 (GBGD) to -17.4 (LCT X GBGD). The cultivars and hybrids exhibited significant differences for leaf water potential during stress and non-stress conditions (Tables 1 and 2). The percent reduction in the leaf water potential during stress was less for LCT (52.3) and LCT x GBGD (71.3) when compared to GBGD (75.3) and GBGD x LCT (119.7). Genotypes showed significant differences for the percent reduction in the leaf water potential during stress period.. The individuals of LCT X GBGD exhibited much variation for the leaf water potential during stress and non-stress conditions (S.D. 2.23 and 2.55 respectively).

RAPD analysis

Forty-five primers detected a total of 515 bands out of which 317 bands were polymorphic (Table 3). The RAPD marker profile of the cultivars and hybrids generated by the primer OPM04 was shown in Plate 1. Jaccard's similarity coefficient based on 515 RAPD markers between all the individuals ranged from 0.539 to 0.947 with a mean of 0.794. The average similarity between the individuals of LCT was 0.785 and between individuals of GBGD was 0.857. In the dendrogram mainly two clusters were observed. Cluster I consisted of the individuals of LCT and LCT X GBGD and the cluster II consisted of GBGD and GBGD X LCT (Fig. 1)

Principal co-ordinates separated the individuals very clearly and it was in correspondence with the UPGMA clustering. The principal co-ordinate one and two explained 76.3 % and 9.3 % of total variation respectively. Tight grouping of individuals of GBGD and GBGD X LCT was clear from the principal co-ordinate plot (Fig.2).

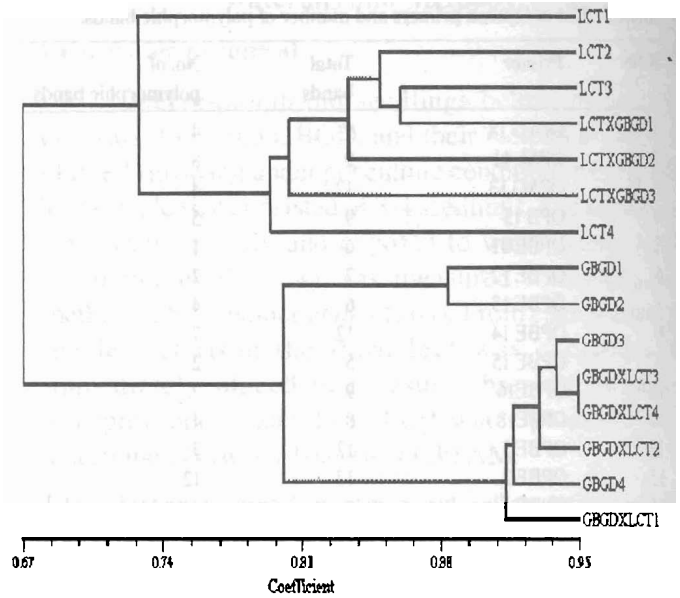


Fig 1. Dendrogram of coconut progeny lines based on RAPD markers

ISSR analysis

Nineteen primers detected a total of 169 bands out of which 108 bands were polymorphic (Table 4). The ISSR banding pattern for the cultivars and hybrids generated by the primer UBC 810 is shown in Plate 2. Jaccard's similarity coefficient based on 169 ISSR markers between all the individuals, ranged from 0.505 to 1.000 with a mean of 0.807. The average similarity between the individuals of LCT was 0.670 and between individuals of GBGD was 0.934. The dendrogram based on the polymorphic markers produced 2 clusters, each comprising individuals of LCT, LCT X GBGD and GBGD and GBGD X LCT (Fig. 3) respectively, but one of the individuals in LCT was lying outside the two

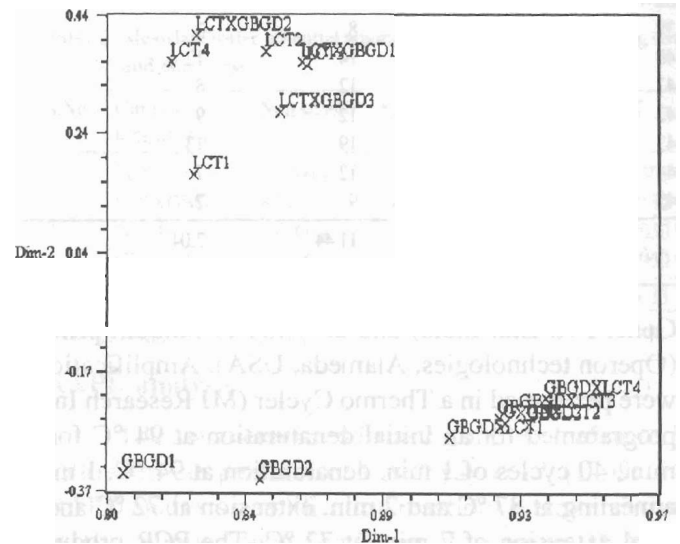


Fig. 2. Principal coordinate analysis of coconut progeny lines based on RAPD markers

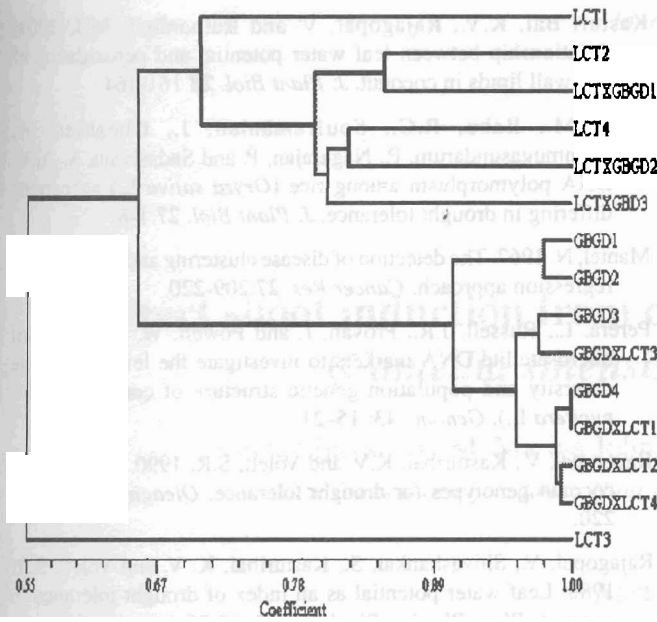


Fig. 3. Dendrogram of coconut progeny lines based on ISSR markers

clusters. The clustering pattern was similar to that of RAPD clustering. The scatter plot based on similarity coefficient (Fig. 4) revealed two groups like RAPD scatter plot. The principal coordinate one and two encompassed 75.7 and 10.8 % of total variation.

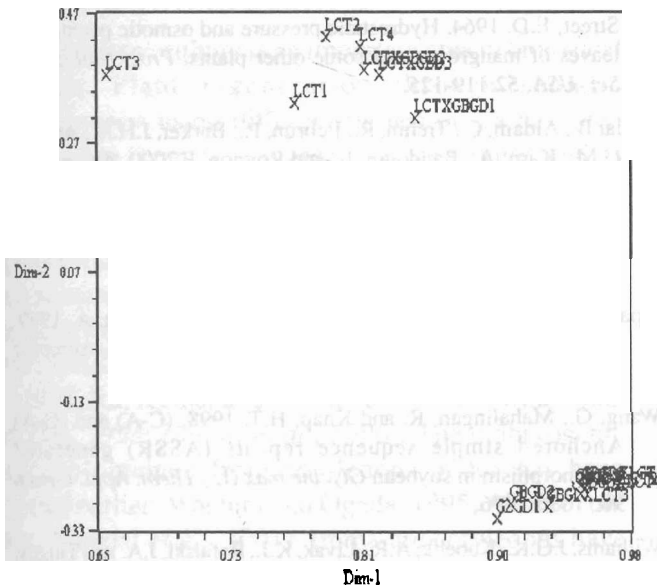


Fig. 4. Principal coordinate analysis of coconut progeny lines based on ISSR markers

Matrix comparison

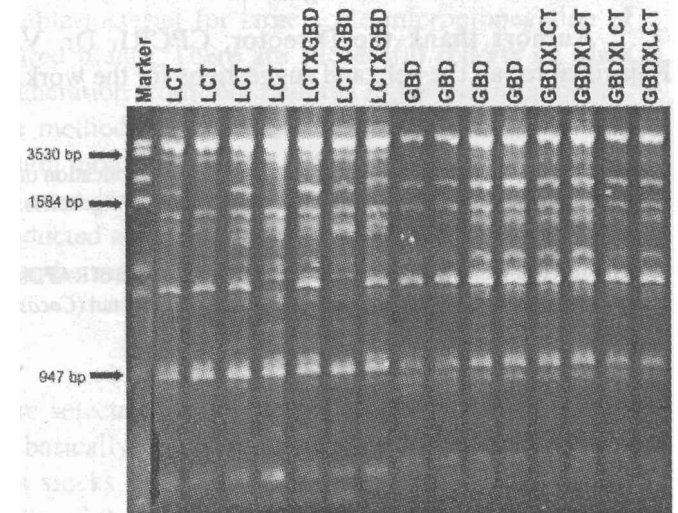
The similarity matrix based on RAPD and ISSR markers were compared and the product moment correlation (r) between RAPD and ISSR was 0.791, which is statistically found to be good fit.

DISCUSSION

Two marker systems (RAPD and ISSR) revealed the genetic structure of the coconut cultivars and hybrids studied. Both RAPD and ISSR markers produced similar clusters. Based on the percent reduction in the leaf water potential, two ranges could be observed. LCT and LCT X GBGD could be categorized into lower range and GBGD (up to 70 %) and GBGD X LCT into higher range (> 70 %). Kasturi Bai *et al.* (2001) had shown significant genotypic differences for leaf water potentials among different coconut cultivars and hybrids.

Both RAPD and ISSR markers were efficient enough to reveal the genetic architecture of coconut. The results obtained from RAPD and ISSR markers were comparable and there was good correspondence between the similarity matrices of RAPD and ISSR markers based on Mantel Z statistics. In many crops including coconut (Teulat *et al.*, 2000), different molecular markers have been compared to reveal the genetic diversity. Both on RAPD and ISSR markers, the average similarity between the individuals of LCT was higher when compared to GBGD. It further emphasized the presence of variation among tall. The individuals of LCT showed much greater variation than GBGD, might be due to its genetic background and it is the fact that tall constitute the heterogeneous population (Perera *et al.*, 2000).

The average similarity based on 169 ISSR markers between the individuals of LCT X GBGD was 0.795 and between individuals of GBGD X LCT was 0.960. This showed that the hybrids of LCT X GBGD exhibited more variation than those of GBGD X LCT combination. When the dwarfs are used as mother palms the hybrids



Marker: λ DNA / EcoRI+HindIII double digest

Plate 1. RAPD marker profile of 15 coconut cultivars and derivatives produced by OPM04

show less variation among themselves and the F_1 s were similar to the mother palm. But the F_1 s from the LCT X GBGD showed variation. GBGD and GBGD X LCT had almost similar DNA banding pattern (Plates 1 and 2), but it varied among them for leaf water potential.

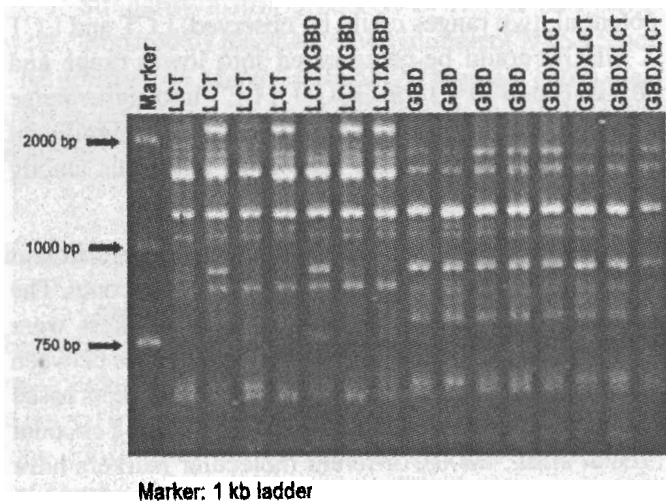


Plate 2. ISSR marker profile of 15 coconut cultivars and derivatives produced by UBC810

This study has reflected the presence of reciprocal differences for genetic variation among the individuals of LCT X GBGD and GBGD X LCT. There was less genetic variation among individuals of GBGD X LCT and GBGD when compared to LCT X GBGD and LCT. The study indicates existence of correlation between leaf water potential and molecular data. Detailed experiments are required to link more physiological traits with molecular markers that will enable Marker Assisted Selection for drought tolerance in coconut.

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