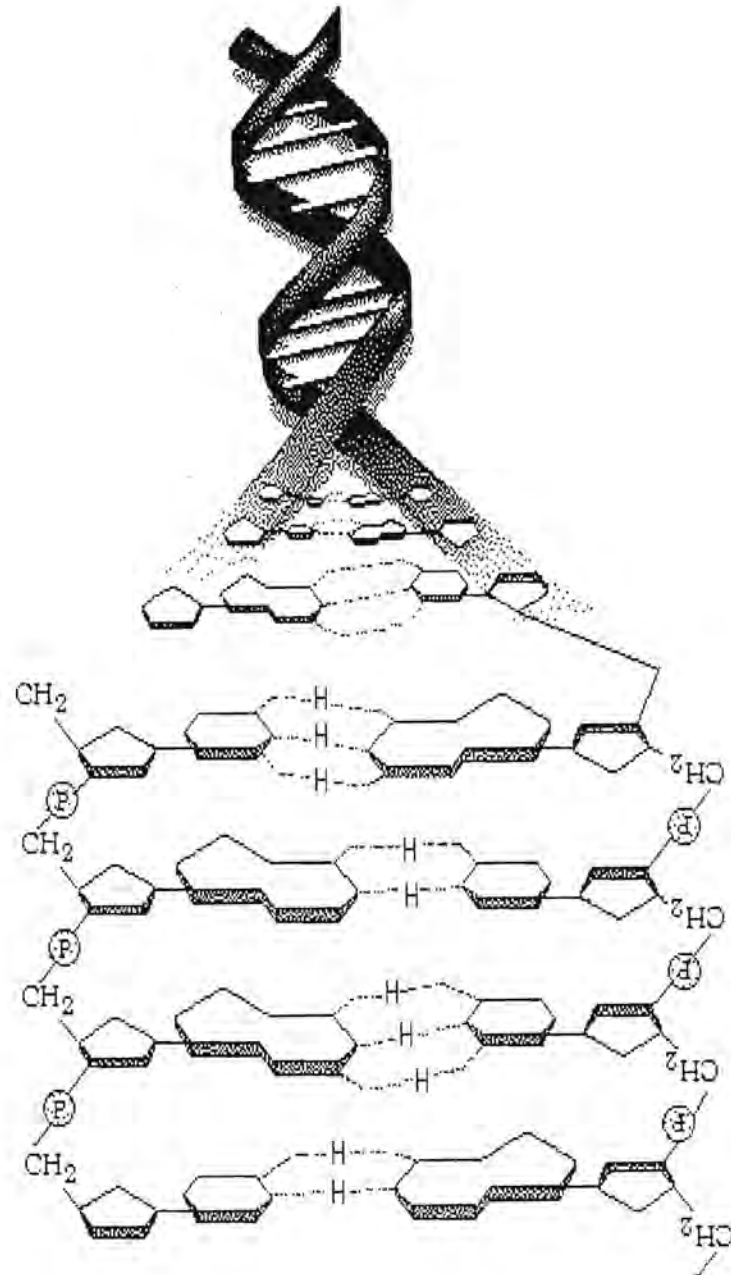# References

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Annex

I) Molecular Methods

I.I   Plant material and DNA extraction

'Medjool' and 'Barhee' plant material originated from 14 plants grown in California that have been propagated solely via off-shoots from mother plant material imported in the beginning of the last century from Northern Africa (Nixon 1950). The *in vitro* plants used were 'Medjool' derived from explant material collected in California, and 'Barhee' derived from explant material collected in the United Arabic Emirates.

Total cellular DNA was isolated from leaves (1 g) using the Nucleon Phytopure Plant DNA extraction kit (Amersham Life Science, UK) according to the manufacturer's instructions. To test the quality and amount of isolated DNA, samples of isolated DNA (1 µl) were run on a 1% agarose gel in TAE buffer (0.04 M Tris-acetate; 1 mM EDTA, pH 8) as outlined by Sambrook et al. (1989). After staining of the gel with ethidium bromide for 15 min, DNA quality was determined on a white/UV-transilluminator, photographed using a Grab-IT system (Vacutec, USA) and the DNA concentration of the samples was visually determined using 4 different λ-phage DNA amounts as standards (25 ng, 50 ng, 100 ng and 250 ng) for comparison.

I.II   Polymerase chain reaction (PCR)

Standard DNA amplifications by PCR were carried out in 25 µl reaction volumes containing 500 mM KCl, 25 mM $MgCl_2$, 100 mM Tris-HCl (pH 8.3), 25 mM of each dNTP and 5 units TaKaRa DNA polymerase (TaKaRa, Japan) in a GeneAmp PCR 9600 system (Perking Elmer, USA). Primers for PCR were obtained either from Operon Technologies (Operon Technologies, USA) or were designed using the online primer design tool of Molecular Biology Shortcuts (MBS), program 'Oligos and Primers' (www.mbshortcuts.com/biotools/index.htlm). The designed primers were then manufactured and purchased from MWG-Biotech AG (Germany). The standard PCR program consisted of 94°C (5 min) to denature double stranded DNA. This was followed by 35 cycles of amplification consisting of denaturing DNA at 94°C (1 min), primer annealing at 55°C or higher depending on primer pair (1 min), and extension of the DNA chain at 72°C (2 min). This was followed by another extension cycle at 72°C for 5 min.

I.III   Representational difference analysis (RDA)

I.III.I   *Preparation of RDA amplicons*

RDA was performed following the general outline described by Lisitsyn et al. (1993). Two micrograms of each of the DNAs ('Barhee' and 'Medjool') were digested with 80 units of the enzyme *Bam*HI or *Hind*III (Amersham Life Science, UK) 100 µg of yeast tRNA (Sigma-Aldrich, USA) was added to the digest to limit non-specific binding of DNA against the sides of eppendorf tubes and pipet tips. The digests were then extracted with an equal volume of phenol/chloroform. The DNA containing upper phase was removed and the DNA precipitated using 1/10 volume 3 M sodium acetate and 2.5 volumes 100% ethanol. The precipitate was washed in 70% ethanol and dried. The digested DNA was dissolved in 18 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA), pH 8, at a concentration of 100 µg/ml.

I.III.II   Ligation of adaptor sequences

One µg of the *Bam*HI and *Hind*III digested DNA were ligated to the adaptor pair RBam 12 and RBam 24 or RHind 12 and RHind 24, respectively. The DNA, primer set (0.6 µM), 10X ligase buffer and water to a final volume of 30 µl were mixed in a microcentrifuge tube. The tubes were placed in a heating block at 55°C and the block was the placed at 4°C until the temperature had fallen to 12°C (60 to 75 min). The tubes were then placed on ice for 3 min, after which 1 µl of T4 DNA ligase (1 unit) (Amersham Life Science, UK) was added and the reaction incubated at 16°C overnight. The ligation reaction was then diluted with 970 µl TE buffer, pH 8, and the amplification reaction was set up as follows:

   80 µl 10X *Taq* Buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl$_2$)
   64 µl dNTP mix (160 mM of each dNTP)
   16 µl (1.0 µM) of one primer of the appropriate pair (J Bam 24 or R Hind 42)
   80 µl of the diluted ligation mixture
   560 µl water

The tube was placed in a heating block at 72°C for 5 min to melt of the 12-mer oligonucleotide. Six µl (30 units) of *Taq* polymerase (TaKaRa, Japan) was added and mixed by pipetting. The mixture was then aliquoted into eighth PCR tubes in a Perkin-Elmer GeneAmp 9600 thermocycler, the block of which was being held at 72°C. After 5 minutes, the thermocycler program run was: 20 cycles: 95°C for 30 sec; 72°C for 2 min, followed by 10 min at 72°C, followed by holding at 4°C.

The 8 tubes were combined an a 10 µl aliquot was run on a 1.5% TAE agarose gel at 100 V for 30 minutes to check the amplification and estimate the concentration by comparison to lambda phage DNA standards. The remainder was extracted with 600 µl phenol/chloroform. The upper phase was removed and the DNA precipitated with 3 M sodium acetate and an equal volume propanol. The precipitate was collected by centrifugation, washed twice with 70% ethanol and dried. The amplicons was redissolved in 80µl TE buffer, pH 8.

I.III.III Removal of the adaptors from amplicons

Of the amplicons that was to be used as driver 150 µg and 10 µg of the tester amplicons were digested with the appropriate enzyme in 800 µl and 200 µl, respectively, at an enzyme concentration of 20 units/µg DNA, at 37°C for one hour. Ten µg Yeast tRNA was added and each digest extracted with phenol/chloroform. The DNA was precipitated with an equal volume of isopropanol and 1/10 volume 3 M sodium acetate, as described above. Isopropanol is used instead of ethanol in order to eliminate very small fragments (e.g. adaptors) from the precipitate. The driver and tester DNAs were both redissolved at approximately 400 µg/ml. Both the digested driver and tester amplicons were run on a 1.5% TAE agarose gel alongside an equal aliquot of undigested amplicons to check the completeness of the digestion. On the same gel, standard lambda phage DNA was run so that the final concentrations of driver and tester DNAs could be estimated and adjusted if necessary.

I.III.IV Change of adaptors on tester amplicons

Tester DNA was prepared by adding a second adaptor pair JBam 12 and 24 for *Bam*HI digested DNA or JHind 12 and 24 for *Hind*III digested DNA to 1 µg of the first

round amplicons in the same way as described for the ligation of the first set. The ligation reaction was then diluted to a final volume of 100 µl with water. An aliquot of the ligate was amplified for 20 cycles in a reaction volume of 20 µl to check that the newly ligated adaptors would support amplification with the new primer.

I.III.V   Subtractive hybridisation and kinetic enrichment

The hybridisation reaction was set up by mixing 80 µl of the driver (~ 40 µg) with 40 µl tester (~ 0.4 µg) amplicons (a ratio of driver : tester of 100 : 1). To this was added 30 µl of 10 M ammonium acetate and 380 µl 100% ethanol. After incubation on ice for 10 min the DNA precipitate was collected by centrefugation for 10 min at full speed in a microcentrifuge. The pellet was washed twice with 70% ethanol and dried after which it was redissolved in 4 µl 3X EE buffer [30 mM N-(2-hydroxyethyl piperizine)-N'-(3-propene sulfonic acid) (EPPS), pH 8, 3 mM EDTA] followed by repeated vortexing interspersed with centrifugation. The solution was collected at the bottom of the tube and overlaid with light mineral oil so that the spherical droplet could be seen to be completely covered by oil. The DNA was denatured by placing it in a heating block at 98°C for 5 minutes. One µl of 5 M sodium chloride solution was added and the tube briefly centrifuged to mix the aqueous phases. The DNA was then incubated at 67°C overnight. Ten µl TE buffer, pH 8, was added and mixed with the aqueous phase, which was then carefully removed from under the oil by pipetting and added to clean microcentrifuge tube containing 380 µl TE buffer and 40 µg tRNA. The appropriately annealed fragments were amplified in the following reaction:

80 µl 10X *Taq* buffer

64 µl dNTP mix (160 mM of each dNTP)

80 µl of the diluted hybridisation mixture

560 µl ds H$_2$O

6 µl *Taq* polymerase (30 units)

The tube was placed in a heating block at 72°C for 5 min to fill in the ends. Sixteen µl of the appropriate primer (the 24mer used in the adapter ligated to the tester amplicons) was added, mixed by pipetting, and the mixture aliquoted into eighth PCR tubes. The tubes were placed in a Perkin-Elmer thermocycler, the block of which, were being held at 72°C.

The following thermocycler program was then run: 10 cycles of 95°C for 30 sec; 72°C for 2 min followed by 10 min at 72°C, followed by holding at 4°C. The tubes were combined and 20 µl of the solution subjected to a further 20 cycles of amplification under the same conditions.

Ten µg of tRNA was added to the remainder of the amplified reaction and then extracted with 600 µl phenol/chloroform. An aliqoute (750 µl) was removed and 75 µl (1/10 volume) of 3 M sodium acetate and 825 µl (one volume) propanol added. The precipitate was collected by centrifugation, after incubation on ice for 10 minutes. The pellet was washed twice with 70% ethanol, dried and dissolved in 40 µl TE buffer, pH 8. At this stage the aliquot subjected to the additional amplification cycles was run on a 1.5% TAE agarose gel to check that amplification occurred. This first round difference product (20 µl) was digested with 20 units of mung bean nuclease (Amersham Life Science, UK) at 30°C for 30 minutes to remove all single- stranded DNA. The reaction was stopped by the addition of 160 µl of TE buffer, pH 8. The digested product was amplified in a reaction mixture consisting of:

80 µl 10X *Taq* buffer

64 µl dNTP mix (160 mM of each dNTP)

16 µl of the same primer used for the 10-cycle amplification

80 µl of the diluted nuclease treated mixture

560 µl ds H_2O

6 µl *Taq* polymerase (30 units)

The mixture was aliquoted into eighth PCR tubes in a Perkin-Elmer thermocycler, the block of which was being held at 72°C. The thermocycler program run was 20 cycles: 95°C for 30 seconds; 72°C for 2 minutes followed by 10 minutes at 72°C, followed by holding at 4°C.

The 8 tubes were combined and a 10 µl aliquot was run on a 1.5% TAE agarose gel at 100 V for 30 min to check the amplification. The remainder was extracted with phenol/chloroform. The upper phase was pipetted into a clean tube and a 1/10 volume of 3 M sodium acetate and 1 volume of propanol added. The tubes were

mixed by inversion and placed on ice for 15 min. The precipitate was collected by centrifugation for 15 min at full speed in a microsentrifuge, washed twice with 70% ethanol and dried. The amplicons were redissolved in 80 µl of TE buffer, pH 8. The concentration was estimated by electrophoresis on a 1.5% TAE agarose gel with lambda phage DNA standards and adjusted to 100 µg/ml.

I.IV   Cloning of the subtraction products

Subtraction products (2 µg) were digested with the 50 units of the appropriate restriction enzyme for 30 minutes at 37°C. One hundred ng of this digest was mixed with 10 ng of pBluescriptII vector (Strategene, USA) digested with the same enzyme (a ratio of insert to vector of 10 : 1). These were extracted with phenol/chloroform and precipitated with sodium acetate and ethanol. The precipitate was collected by microcentrifugation and the pellet washed twice with 70% ethanol before being dried. The dried pellet was redissolved in 8 µl water. To this was added 1 µl 10X ligase buffer and 1 µl T4 ligase, and the mixture incubated overnight at 16°C. Two µl of the ligate was used to transform competent XL1Blue cells and 50 plasmid-containing colonies carrying an insert were selected and probed with either the 'Barhee' or 'Medjool' labeled driver amplicons using the *Gene Images* random prime-labeling module (Amersham Life Sciences UK). Ten colonies that showed a much stronger signal after hybridization with the 'Barhee' amplicons than with the 'Medjool' amplicons were selected for plasmid isolation and determination of the insert sequence and size.

I.V   Southern-blot hybridization

Total genomic DNA of 'Barhee' and 'Medjool' (1 µg) were digested with two units of each of the restriction enzymes *Bam*HI and *Eco*RI at 37°C for two hours. The restricted DNA was then separated on a 1.5% TAE agarose gel using electrophoresis, and transferred to a positively charged nylon membrane (Roche, Switzerland), as described by Sambrook et al. (1989). Cloned fragments of Dp41 and Dp36 were used as probes, which were labeled using the *Gene Images* random prime, labeling module. Membranes were pre-hybridized and hybridized at 65°C in a hybridization buffer containing 5% SSC, 0.1% SDS and 20-fold dilution of the liquid

block provided. Hybridization was carried out overnight and the membranes washed at 60°C using a 1% SSC and 0.1% SDS solution. Detection was preformed using the *Gene Images CDP-Star* detection module (Amersham Life Sciences UK), according to the manufacturer's instructions. Membranes were exposed to Hyperfilm ECL (Amersham Life Sciences UK) and the films developed.

I.VI   Sequence analysis

DNA sequence analysis was carried out with the dideoxy chain terminator method developed by Sanger et al. (1997). Recombinant plasmids were sequenced using fluorescent dye terminators and AmliTaq from the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Inc., Foster City, Calif.), in a cycle sequence protocol according to the recommendations of the manufacturer. Sequence reactions were run on PAGE (polyacrylimide gel electrophoresis) using an ABI PRISM™ 377 Autosequencer (Perkin-Elmer Applied Biosystems). Sequence electropherograms were analysed using Sequence Navigator version 1.0.1 (Perkin-Elmer).

II) Tissue culture and somatic embryogenesis

II.I Tissue culture initiation

The most responsive embryo developmental stages for somatic embryogenesis initiation range from the cleavage polyembryony, to the early (precotyledonary) dominance stage (Percy *et al.*, 2000). For culture initiation, seeds were extracted from developing cones and placed in Petri dishes containing moistened filter paper. Within 1 hour of extraction, seeds were surface sterilized in preparation for dissection. Seeds were surface sterilised by placing them in metal baskets and stirring vigorously for 6 min in 200 ml of 3% (v/v) hydrogen peroxide with one drop of Tween 20. Seeds were then rinsed tree times for 2 min each with sterile water and transferred to a petridish containing a moist, sterile filter paper. The seed coat, nucellus, and membrane surrounding the metagametophyte were removed under a stereomicroscope, and the megagametophyte was placed on initiation medium. Five to eight megagametophytes were cultured in each Petri dish containing approximately 25 ml of medium, and dishes were sealed with parafilm. Explants were cultured in darkness at 24°C for 16 weeks and examined every 4 weeks for embryogenic tissue initiation.

II.II Embryogenic tissue proliferation and culture maintenance

Explants displaying proliferation from the zygotic embryo cells were microscopically selected, then embryogenic tissue was removed, dispersed by vigorous shaking in liquid MLV maintenance medium, poured onto a sterile filter paper disk (Whatman No. 2, 5.5 cm) in a Büchner funnel and a short low-pressure pulse (55 kPa) was applied to drain the medium prior to transferring the disc to fresh medium. This approach was also used for maintenance of embryogenic tissue. Maintenance and proliferation cultures were incubated in darkness at 24°C.

II.III Maturation of somatic embryos

The somatic embryo experiments were performed by combining embryogenic tissue of one line (from several plates), one week after subculture, in a 50 ml test tube,

adding liquid media without growth regulators and vigorously shaking the tube to break up the clumps of tissue into a fine suspension. Subsequently 3 ml containing approximately 0.2 g of suspended embryonal masses were withdrawn with a wide-mouth pipette and placed on a moist filter paper disk (Whatman No. 2, 5.5 cm in diameter) in a Büchner funnel attached to a vacuum pump. A short low-pressure pulse (5 sec, 55 kPa) was applied to drain all liquid medium and anchor the embryonal masses to the filter paper as a thin layer. Each disk of filter paper with the embryonal masses was subsequently placed on maturation medium in a 10 X 20 mm Petri dish and cultured for up to 10 weeks. The cultures were kept under dim light condition at 1.6 μmol m^{-2}s^{-1} from cool white florescence lamps (Philips F72T12/CW, 65 W) under a 16 h photoperiod at 24°C.

II.IV Tissue culture media

II.IV.I MLM 10X stock

	2 Liters	Final concentration in medium [g/L]
NH$_4$NO$_3$	16.5 g	0.825
KNO$_3$	19.0 g	0.95
MgSO$_4$.7H$_2$O	18.5 g	0.925
KH$_2$PO$_4$ (monobasic)	3.4 g	0.17
CaCl$_2$.2H$_2$O	0.22 g	0.011
LM Minor stock (100X)	200 ml	
LM Vitamin stock (100X)	200 ml	
Myo-Inisitol	2.0 g	0.1
Iron Solution	100 ml	
dH$_2$O to	2 L	
Stored at -20°C or 2 weeks at +4°C		

II.IV.II   MLM Minor Stock 100X

	1 Liter	Final concentration in medium [g/L]
$Na_2MoO_4.2H_2O$	0.125 g	0.00125
KI	0.415 g	0.00415
H_3BO_3	3.1 g	0.031
$MnSO_4.H_2O$ (or $MnSO_4.4H_2O$)	2.1 g (2.7 g)	0.021 (0.027)
$ZnSO_4.7H_2O$	4.3 g	0.043
$CuSO_4.5H_2O$	0.05 g	0.0005
$CoCl_2.6H_2O$	0.013	0.00013
DH_2O to	1 L	
Stored at -20°C		

II.IV.III   MLM Vitamin stock 100X

	1 Liter	Final concentration in medium [g/L]
Nicotinic acid	0.05 g	0.0005
Pyridoxine.HCL	0.01 g	0.0001
Thiamine.HCL	0.01 g	0.0001
dH_2O to	1 L	
Stored at -20°C		

II.IV.IV   MLM Iron solution

	100 ml	Final concentration in medium [g/L]
Na_2EDTA	0.746 g	0.0373
$FeSO_4.7H_2O$	0.556 g	0.0287
dH_2O to	100 ml	
Made fresh		

II.V   Growth regulators

II.V.I   2,4-Dichlorophenoxyacetic Acid (2,4-D) stock (1 mg/ml)

2,4-D	100 mg
95% Ethanol	50 ml
DH$_2$O to	100 ml
Stored at +4°C	

II.V.II   6-Benzyl-Aminopurine (BA) stock (2.5 mg/ml)

BA	50. mg
Warmed 0.5 N HCL (or NaOH)	5 ml to dissolve
Warmed dH$_2$O to	100 ml
Stored at +4°C	

II.V.III   (±) cis-trans Abscic (ABA) stock (10mM)

ABA	0.02643 g
1 N NaOH	drops to dissolve
dH$_2$O to	10 ml
Filter sterilized and wrapped in foil. Made fresh.	
1 ml stock/ L = 10 μM	

II.V.IV   Glutamine stock (25 mg/ml) (Amino acids)

Glutamine	25.0 g
dH$_2$O to	1 L
pH 5.8. Filter sterilized. Stored frozen	

II.VI   MLV Media

II.VI.I   MLV Ultra low medium (MLV UL)

		1 Liter
MLM 10X stock		100 ml
Casein Hydrolosate (Casamino Acids)	1 g/L	1.0 g
Sucrose	2 %	20.0 g
2,4-D (1 mg/ml stock)	1.1 μM	0.25 ml
BA (0.5 mg/ml stock)	1.1 μM	0.5 ml
dH$_2$O to		1 L
Phytagel	0.4 %	4 g
Set pH to 5.7. Autoclaved		
Glutamine (25 mg/ml sterile stock)	0.5 g/L	20 ml

II.VI.II   MLV Standard medium (MLV Std)

		1 Liter
MLM 10X stock		100 ml
Casein Hydrolosate (Casamino Acids)	1 g/L	1.0 g
Sucrose	2 %	20.0 g
2,4-D (1 mg/ml stock)	9.5 μM	2 ml
BA (0.5 mg/ml stock)	4.4 μM	2 ml
dH$_2$O to		1 L
Phytagel	0.4 %	4 g
Set pH to 5.7. Autoclaved		
Glutamine (25 mg/ml sterile stock)	0.5 g/L	20 ml

II.VI.III   Maturation medium (MM)

		1 Liter
MLM 10X stock		100 ml
Casein Hydrolosate (Casamino Acids)	1 g/L	1.0 g
Sucrose	6 %	60.0 g
dH_2O to		1 L
Phytagel	1 %	10 g
Set pH to 5.7. Autoclaved		
Glutamine (25 mg/ml sterile stock)	0.5 g/L	20 ml
ABA (10 mM sterile stock)	120 μM	12 ml

III   General solutions and buffers

III.I   Antibiotics

III.I.I   Ampicillin stock solution (50 mg/ml)

Dissolve 2 g Ampicillin (D(-)-a-Aminobenzylpenicillin sodium salt) (Sigma-Aldrich, Germany) powder in 40 ml of sterile distilled water. Aliquote in 1.5 ml tubes and store at -20°C.

III.I.II   Tetracycline stock solution (10 mg/ml)

Dissolve 0.1 g of tetracycline powder (Sigma-Aldrich, Germany) in 5 ml 100% ethanol, adjust volume to 10 ml with sterile distilled water. Cover tube with foil and store at -20°C.

III.II   Southern blotting solutions

III.II.I   Neutralization buffer

Combine 121.1 g of Tris base (Sigma-Aldrich, Germany) and 87.7 g NaCl. Add 900 ml distilled water. Adjust pH to 8.0 with concentrated HCl. Adjust volume with distilled water to 1 L and autoclave.

III.II.II   Denaturing buffer

Combine 87.7 g NaCl and 20 g NaOH. Add distilled water to 1 L. Stir to dissolve and autoclave.

III.II.III 20X Standard Saline-Citrate (SSC)

Combine 174.4 g NaCl, 88.3 g $C_6H_5Na_3O_7.2H_2O$ (Sodium-citrate) in 800 ml distilled water. Stir to dissolve and adjust volume to 1 L. Autoclave.

III.III Buffers and salt solutions

III.III.I Agarose gel buffer (TAE) (50X stock)

Combine 968 g Tris base, 228.4 ml glacial acetic acid and 400 ml 0.5 M EDTA, pH 8.0. Add 3 L distilled water and stir until solids dissolve. Adjust volume to 4 L with distilled water. Dilute to 1X before use.

III.III.II Agarose gel loading buffer (6X)

Combine 0.063 g bromophenol blue (BPB), 0.063 g xylene cyanol FF (XC) and 2.5 ml glycerol. Add distilled water to 25 ml. Aliquot into 1.5 ml tubes and store at -20°C. Add 1 μl to sample before loading onto agarose gel.

III.III.III Ethidium Bromide solution

Dissolve 0.2 g ethidium bromide powder in 20 ml distilled water. Vortex to dissolve and cover container with foil.

III.III.IV 5 M Ammonium acetate (NH_4OAc)

Dissolve 385.4 g ammonium acetate in 500 ml distilled water by slowly adding the ammonium acetate powder into the stirring water. Adjust volume to 1 L. Autoclave and store at RT.

III.III.VI   3 M Sodium acetate (NaOAc) pH 4.8

Dissolve 40.82 g NaOAc.3H$_2$O in 80 ml distilled water and stir on a hot plate until dissolved. Adjust pH to 4.8 with glacial acetic acid. Adjust volume to 100 ml with distilled water. Autoclave and store at RT.

III.III.VII   5 M Sodium chloride

Dissolve 29.22 g NaCl in 80 ml distilled water. Adjust volume to 100 ml, autoclave and store at RT.

III.III.VIII   1 M Tris-HCl pH 8.0

Dissolve 12.11 g Tris base in 80 ml of distilled water. Adjust pH to 8.0 using concentrated HCl. Adjust volume to 100 ml with distilled water. Autoclave and store at RT.

III.III.IX   0.5 M EDTA pH 8.0

Dissolve 8 g NaOH pelles in 400 ml distilled water. Add 93.05 g Na$_2$EDTA.2H$_2$O and allow to dissolve. Adjust pH to 8.0 using more NaOH pellets. Adjust volume to 500 ml with distilled water. Autoclave and store at RT.

III.III.X   TE buffer (10 mM Tris-HCL, 1 mM EDTA)

Combine 1 ml 1M Tris-HCl pH 8.0 and 0.2 ml 0.5 M EDTA pH 8.0. Adjust volume to 100 ml with distilled water. Autoclave and store at RT.

III.III.XI   Low TE buffer (10 mM Tris-HCL, 0.1 mM EDTA)

Combine 1 ml 1M Tris-HCl pH 8.0 and 20 µl 0.5 M EDTA pH 8.0. Adjust volume to 100 ml with distilled water. Autoclave and store at RT.
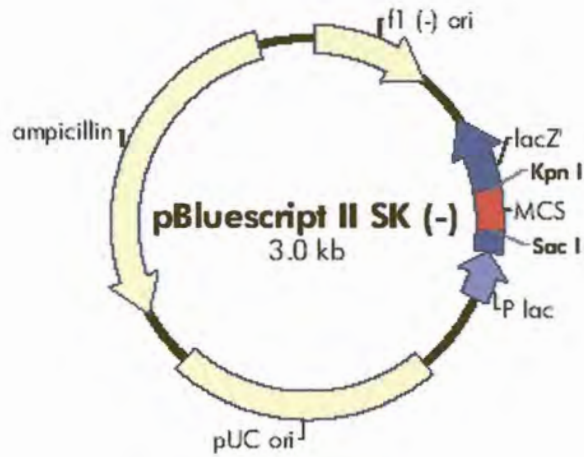
III.III.XII   <u>10 N Sodium hydroxide (NaOH)</u>
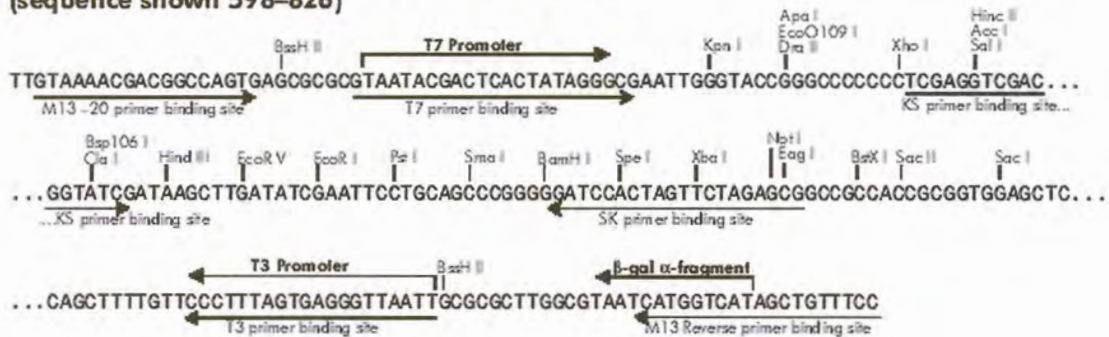
Dissolve 40 g NaOH pellets in 70 ml distilled water. Adjust volume to 100 ml. Store at RT.

IV.   Plasmid maps

IV.I   pBluescript II (Stratagene USA)

pBluescript II SK (+/–) Multiple Cloning Site Region
(sequence shown 598–826)

IV.II   pMosBlue (Amersham UK)

pMOS*Blue*
(2887bp)

Hind III (45)
BspM (50)
Sph I (58)
Sse8387 I (61)
Pst I (61)
Sal I (63)
Acc I (64)
Hinc II (65)
Xba I (69)
Spe I (83)
Nde I (88)
EcoR V (95)
BamH I (99)
Ava I (104)
Sma I (106)
Kpn I (112)
Sac I (116)
EcoR I (120)

Dra III (503)
BsaA I (509)
Drd II (513)
Bcc I (515)
Nae I (611)

T7 start (41)

lacZ

f1 origin

Sap I (2863)

Afl III (2846)

ori

MlwN I (2137)

HgiE II (1964)

Ap

Xmn I (1099)
BsaH I (1119)
Sca I (1177)

Earn1105 I (1658)
Pfl1108 I (1639)
Bsa I (1505)
Gsu I (1440)

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V. Primer sequences

RAPD and SCAR primers	
OPE-01	5'-CCCAAGGTCC-3'
OPE-06	5'-AAGACCCCTC-3'
DpSL	5'- GTGTTAGGGGCAAAATGGAA–3'
DpSR	5'- TTGTCCGTCTGAGACTCCCT–3'
Date palm subtraction product specific primers	
DP36L	5'-CTATCGACGACAGGCTGACA -3'
DP36R	5'-GACCCGGACTTGTTGGAGTA-3'
DP41L	5'-CCTTCTCCCCGTAGTAACCG-3'
DP41R	5'-AGGAAAGGCAACCTACCGAG-3'
DP50L	5'-TACACGATGTCCCTCAACCA-3'
DP50R	5'-GGAACATTTCCTCGGTATCC-3'
PLM 1	5'-TTACAGAGGGGAAAGGAGGA-3'
PLM 4	5'-GGAAGGAGGTGGCTCCG-3'
PLB11	5'-CGCAATCTTGCAAGTATCAGT-3'
RDA adapters	
R Bam 24	5'-AGCACTCTCCAGCCTCTCACCGAG-3'
R Bam 12	5'-GATCCTCGGTGA-3'
J Bam 24	5'-ACCGACGTCGACTATCCATGAACG-3'
J Bam 12	5'-GATCCGTTCATG-3'
N Bam 24	5'-AGGCAACTGTGCTATCCGAGGGAG-3'
N Bam 12	5'-GATCCTCCCTCG-3'
R Hind 24	5'-AGCACTCTCCAGCCTCTCACCGCA-3'
R Hind 12	5'-AGCTTGCGGTGA-3'
J Hind 24	5'-ACCGACGTCGACTATCCATGAACA-3'
J Hind 12	5'-AGC TTGTTCATG-3'
N Hind 24	5'-AGGCAGCTGTGGTATCGAGGGAGA-3'
N Hind 24	5'-AGCTTCTCCCTC-3'

R Hpa 24	5'-AGCACTCTCCAGCCTCTCACCGAC-3'
R Hpa 11	5'-CGGTCGGTGAG-3'
J Hpa 24	5'-ACCGACGTCGACTATCCATGAAAC-3'
J Hpa 11	5'-CGGTTTCATGG-3'
N Hpa 24	5'-AGGCAACTGTGCTATCCGAGGGAC-3'
N Hpa 11	5'-CGGTCCCTCGG-3'
S Hpa 24	5'-ACTTCTACGGCTGAATTCCGACAC-3'
S Hpa 12	5'-CGGTGTCGGAAT-3'
Sequencing primers	
T7	5'-TAATACGACTCACTATAGGG-3'
Sp6	5'-CATACGATTTAGGTGACACTATAG-3'

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Use of representational difference analysis for the characterization of sequence differences between date palm varieties

Abstract Representational difference analysis was applied to subtract the genomes of the two date palm varieties, Barhee and Medjool, for identification and characterization of unique genome differences suitable for discriminating between individual plants and the two varieties. Three different DNA difference products were isolated from Barhee representing families of dispersed, repeated variable sequences present in the genome of both varieties. Several variant members of repeated DNA were detected by sequence analysis, containing base changes from C to T and G to A and short deletions. Mutated DNA sequences could be amplified in a polymerase chain reaction-based test from a much smaller number of Barhee plants than from Medjool plants allowing the differentiation between individual plants and partial discrimination between varieties.

Keywords Date palm · Representational difference analysis · Genome analysis · Hyper-variable DNA · Plant variety identification

Introduction

Genomes of closely related plants or varieties can differ by only a few coding genes or in minor genome re-organizations and a range of different approaches is available for detection of such genetic differences. Among these are the analyses of r-DNA intergenic regions (Scribner

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South Africa

and Pearce 2000) and of simple sequence repeats (SSRs), which are also known as microsatellites. SSRs have been used for example for identification of varieties of *Chrysanthemum* and *Citrus* (Wolff et al. 1995; Fang and Roose 1997) as well as individual plants of oilseed rape cultivars (Charters et al. 1996). However, the two most widely used genetic techniques to detect plant variation are random amplified polymorphic DNA (RAPD) analysis, which detects DNA polymorphisms amplified by arbitrary primers (Williams et al. 1990; Welsh and McClelland 1990) and amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995; O'Hanlon et al. 2000). Recently, the RAPD technique has also been applied to identify date palm on the variety level (Corniquel and Mercier 1994; Sedra et al. 1998). All these techniques are useful for the differentiation of plants by identifying random polymorphisms. However, the comparisons are usually made on the basis of the presence or absence of a band rather than directly on any DNA sequence variation.

In contrast, representational difference analysis (RDA) allows the cloning and sequencing of fine genome differences between two highly similar genomes and further provides exact sequence information about these differences. RDA detects any kind of labile DNA region in two genomes to be compared and can be used to derive probes for genomic losses, rearrangements, amplifications, point mutations and pathogenic organisms found within any of the genomes compared (Lisitsyn et al. 1993, 1994; Michiels et al. 1998). RDA has been applied in a variety of contexts including the isolation of repetitive sequences present in only one of the compared genomes (Navin 1996). Nekrutenko et al. (2000) used RDA to create a species-specific marker for voles and Toder et al. (2001) have applied RDA in evolutionary genomics to search for overall genome differences between humans and the great apes. RDA has also been used to determine differences between two distantly related oak species where similarities of isolated RDA fragments with known retrotransposons were found (Zoldos et al. 2001). In addition, Donnison et al. (1996)

applied RDA to identify male-specific restriction fragments in the dioecious plant *Silene latifolia*. RDA has also been used to identify polymorphisms in banana lines that are a result of genomic rearrangements during in vitro propagation resulting in markers useful for the detection of early variation in the initiation of tissue culture plants (Cullis and Kunert 2000). One of the specific advantages of RDA is that subtractions between pooled DNA samples can be performed in order to identify specific polymorphisms that are only present in either a particular individual or a particular variety rather than relying on identification based on a pattern of polymorphic bands.

The aim of this study was to apply the RDA technology to date palm and to investigate if it is a useful technique for generation of markers suitable for identification and characterization of variable regions in the date palm genome. Such variable regions might ultimately be applicable for either individual date palm plant or variety identification/discrimination. In general, there is a need for DNA-based markers in date palm that will facilitate variety identification as well as confirmation of the genetic fidelity of individual propagated plants. The current identification based on morphological characteristics is often difficult. For example, in the Kingdom of Saudi Arabia alone almost 400 date palm cultivars have been classified (Hussain and El-Zeid 1978), based mainly on their fruit characteristics that are expressed in the mature stage of the plant. In this study, plants of the two date palm varieties Barhee and Medjool, which are commonly used in commercial tissue culture for the asexual production of date palm via somatic embryogenesis, have been investigated. A repetitive labile DNA region has been identified allowing the differentiation between individual plants and partial discrimination between varieties.

Materials and methods

Plant material and DNA extraction

The date palm varieties Medjool and Barhee were used as the source of DNA. The in vitro plants used were Medjool derived from explant material collected in California, and Barhee derived from explant material collected in the United Arabic Emirates. Non-tissue culture-derived Medjool and Barhee plant material originated from 14 plants grown in California that have been propagated solely via off-shoots from mother plant material imported in the beginning of the last century from Northern Africa (Nixon 1950). Total cellular DNA was isolated from leaves (1 g) using either the technique outlined by Aitchitt et al. (1993) or the Nucleon Phytopure plant DNA extraction kit (Amersham Life Sciences, UK) according to the manufacturer's instructions. Both techniques gave similar results.

Preparation of RDA amplicons

RDA was performed following the general outline described by Lisitsyn et al. (1993). Two micrograms of each of the DNAs (Barhee and Medjool) were digested with 80 units of the enzyme *Bam*HI or *Hin*dIII. The digests were then extracted with phe-

nol/chloroform, precipitated and resuspended at a concentration of 100 μg/ml. The *Bam*HI and *Hin*dIII digests were ligated to the adaptor pair JBam 12 (5′-GATCCGTTCATG-3′) and JBam 24 (5′-ACCGACGTCGACTATCCATGAACG-3′) or RHind 12 (5′-AGCTTGCGGTGA-3′) and RHind 24 (5′-AGCACTCTCCA-GCCTCTCACCGCA-3′), respectively. The ligation products were amplified by polymerase chain reaction (PCR) by using the primer JBam 24 or RHind 24 to generate the first-round amplicons, followed by digestion with *Bam*HI or *Hin*dIII to remove the adaptors. Tester DNA was prepared by adding a second adaptor pair NBam 12 and 24 (5′-GATCCTCCCTCG-3′ and 5′-AGGCA-ACTGTGCTATCCGAGGGAG-3′) for *Bam*HI-digested DNA or JHind 12 and 24 (5′-AGCTTGTTCATG-3′ and 5′-ACCGAC-GTCGACTATCCATGAACA-3′) for *Hin*dIII-digested DNA to the ends of the first-round amplicons.

Subtractive RDA hybridization, kinetic enrichment and cloning of the difference products

The hybridization reaction was set up using 40 μg driver DNA (Medjool) and 0.4 μg tester DNA (Barhee) (100:1 driver/tester ratio) in a final volume of 4 μl hybridization buffer consisting of 30 mM EPPS [(2-hydroxyethyl piperizine)-*N*′-(3-propene sulfonic acid)], pH 8, and 3 mM EDTA. The DNA was denatured at 100°C for 10 min, 1 μl of sodium chloride (5 M) was added to a final concentration of 1 M and the reaction incubated at 67°C for 16 h. The hybridization mix was then diluted and an aliquot amplified using NBam 24 or JHind 24. The first round of amplification was for ten cycles, followed by digestion of the products by mung bean nuclease. The nuclease-treated product was then amplified for an additional 20 cycles. The resulting amplicons, which are called the first difference product, were used in this study. These subtraction products were digested with the appropriate restriction enzyme and ligated into the appropriately digested pBluescript II (Strategene, USA). The ligation products were transformed into XL1Blue-competent cells and 50 plasmid-containing colonies carrying an insert were selected and probed with either the Barhee- or Medjool-labeled driver amplicons using the Gene Images random prime-labeling module (Amersham Life Sciences). Colonies that showed a much stronger signal after hybridization with the Barhee amplicons than with the Medjool amplicons were selected for plasmid isolation and determination of the insert sequence and size.

Primer design and testing

Pairs of primers were designed using a standard design program (Expasy, Switzerland). The primer pairs were used in a PCR reaction using Barhee and Medjool DNA as template at various annealing temperatures to optimize the PCR reaction. The PCR reactions were carried out in 25-μl volumes containing 25 ng total genomic DNA, 15 ng primer, 100 mM of each dNTP, 10 mM TRIS-HCl, pH 8.3, 2 mM MgCl₂ and 0.5 units Taq polymerase (Takara, Japan). Amplification was performed using a Perkin Elmer Gene-Amp PCR system 9600 with the following program: (1) 94°C for 5 min×1 cycle; (2) 94°C for 1 min, 65°C or 60°C for 1 min depending on the primer pair, 72°C for 1 min×35 cycles; (3) 72°C for 5 min×1 cycle, and optional soak period at 4°C. The products were separated on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

Southern blot hybridization

Genomic DNA was digested with different restriction enzymes, separated on a 1.5% TAE agarose gel using electrophoresis, and transferred to a positively charged nylon membrane (Roche, Switzerland), as described by Sambrook et al. (1989). Cloned bands were used as probes and were labeled using the Gene Images random prime labeling module (Amersham Life Sciences). Mem-

Fig. 1 Sequence analysis of isolated RDA clones DP2, DP36 and DP41 and position of the most variable DNA region of the DP41 clone

```
DP-2    CCTATCGAAC CCATTCATAC AGAGCCAGTT TTCAATGTCC CTCAACCATC GCGCGGATCT AGTAGGGTCT CCCATCCTCC  80

DP-36   CCTATGGCGA CCGGCTGACC TGGCACTGGT GTCCGCAGCA ACTCTGCTCG GATGGAAAGA AGTCGACCTC GACGAAAGCG  80

DP-41   CCTTCTCCCC GTAGTAACCG GCCTCCCCGC AAATCTTGCA AGTATCACTG AGGGGGAAGA AGGAGGAGGG GCCTCCGCAC  80
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CGATAGATAC TTAGGTATAC TAGAAGAGGA TACCGAGAAA ATGTTCCTAG TGGGAGATAG AGATCACATAC AGGAT  156

GCTCGGTAAA GCCCCGGTAG TACTCCAACA AGTCCGGGTC AATCCGACGG TATCTCCTCG CGCTGGAT  147

GTGCCTCACG TCGTGGGGAC ACCGTAGATG GCTCGGTAGG TTGCCTTTCC TCCGTTGGAT  141
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branes were pre-hybridized and hybridized at 65°C in a buffer containing 5× SSC, 0.1% SDS and a 20-fold dilution of the liquid block provided overnight and washed at 60°C using a 1× SSC and 0.1% SDS solution. Detection was performed using the Gene Images CDP-Star detection module, according to the manufacturer's instructions. Membranes were exposed to Hyperfilm ECL (Amersham Life Sciences) and the films developed.

Sequence analysis

For plasmid sequencing, PCR products were cloned into the plasmid pMOS*Blue* (Amersham Pharmacia Biotech, UK) and plasmids were recovered from transformed MOS*Blue* cells selected on an appropriate antibiotic. Sequencing was performed using Sequenase (Perkin Elmer, USA) according to the manufacturer's instructions on an automated DNA sequencer (Applied Biosystems, USA).

Table 1 Sequences of primers used to amplify different parts of clone DP41 with DP41R 5'-GCAACCTACCGAGCCATCT-ACGGT-3' as the right-hand primer and the difference product DP36 with DP36R 5'-GGAACATTTTCTCGGTATCCTC-3' as the right-hand primer, and a total number of seven non-tissue culture-derived plants amplifying a DNA fragment with the predicted size. *Numbers in parentheses* indicate the total number of plants tested

Primer	Sequence (5'–3')	Amplification product	
		Barhee	Medjool
DP41L	CCTTCTCCCCGTAGTAACCG	5 (7)	7 (7)
PLM1	TTACAGAGGGGAAAGGAGGA	5 (7)	7 (7)
PLM4	GGAAGGAGGTGGCTCCG	1 (7)	7 (7)
PLB11	CGCAATCTTGCAAGTATCAGT	2 (7)	6 (7)
DP36L	TCGAACCCATTCATACAGAGC	4 (7)	7 (7)

Results

The subtractions between Barhee and Medjool were performed with either Barhee as tester and Medjool as driver or with Medjool as tester and Barhee as driver for amplicons derived from *Bam*HI- or *Hin*dIII-digested DNAs. Following a single round of subtraction using a tester to driver ratio of 1:100 only one of the four subtractions, that with Barhee *Bam*HI-digested DNA as tester and Medjool as driver, produced a DNA difference product, which is approximately 150 bp in length. The difference product was cloned and 50 *Escherichia coli* colonies containing the cloned difference product (data not shown) hybridized separately with labeled Barhee tester and Medjool driver amplicons. Both sets of amplicons hybridized to the colonies indicating that the isolated difference product or a closely related sequence was present in both varieties.

Ten *E. coli* colonies with the greatest differential signal between tester and driver hybridization were selected, plasmid DNA isolated and the insert sequence determined. This analysis revealed that the cloned difference product consisted of at least three sequences with lengths of 141 bp (DP41), 147 bp (DP36), and 156 bp (DP2) (Fig. 1) indicating a complex mixture of fragments in the difference product. Seven of the sequenced inserts were identical to DP2 and two identical to DP36. The DP41 sequence contained an AGG motif repeated in tandem 3 times. A search of these three sequences using different databases and DNA sequence analysis tools, such as Blast, FastA and the Smith-Waterman algorithm, result-

ed in a 75% homology to *Oryza sativa* genomic DNA, chromosome 1 (accession no. AP002902) when a local alignment was done with DP41 but no homology was found for DP2 or DP36.

Since most of the sequenced clones were identical to DP2, we selected only the clones DP36 and DP41, the latter of which was unique, for further characterization. Hybridization of labeled DP41 to *Eco*RI- or *Bam*HI-digested DNAs from tissue culture-derived Medjool and Barhee gave a smear pattern, which is characteristic of a dispersed, repetitive sequence (data not shown). Similar hybridization profiles were obtained when either DP36 or DP2 were used as probes.

Four primers, DP41L and DP41R and DP36L and DP36 R (Table 1), were designed from the DP41 and DP36 sequences. These primer pairs amplified the predicted 120-bp or 110-bp PCR product, respectively, from genomic DNA of six different tissue culture-derived Medjool and Barhee plants but failed to amplify a fragment in two non-tissue culture-derived Medjool and three non-tissue culture-derived Barhee plants (Fig. 2; Table 1). The DP41 amplification product was then characterized in more detail. From each of the six tissue-culture-derived Barhee and Medjool plants, three independent clones of the DP41 amplification product were sequenced (Fig. 3). These 36 clones from different Medjool and Barhee plants revealed a high degree of homology with DP41, the differences being minor base pair changes or single base deletions occurring mainly in a
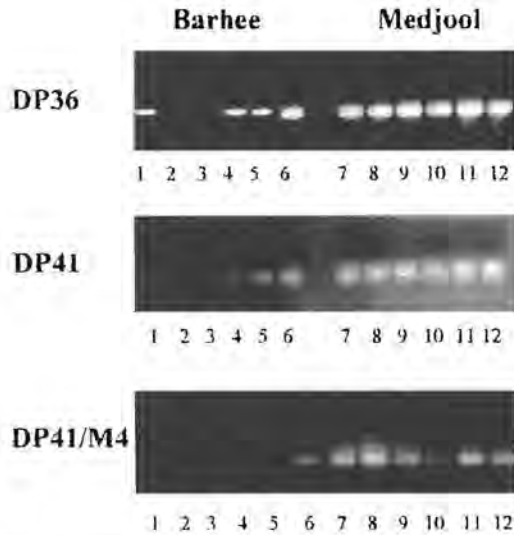
274

Barhee   **Medjool**

DP36

1 2 3 4 5 6   7 8 9 10 11 12

DP41

1 2 3 4 5 6   7 8 9 10 11 12

DP41/M4

1 2 3 4 5 6 7 8 9 10 11 12

Fig. 2 PCR products of genomic DNA from six out of seven Barhee (B) and Medjool (M) plants amplified with primers for DP36, DP41 and DP41/M4 with the following template DNAs: *lanes 1–6* non-tissue culture-derived B plants; *lanes 7–12* non-tissue culture-derived M plants

```
DP41  TCTTGCAAGTATCACTGAGGGGGAAGAAGGAGGAGGGGCCTCCGC
M1    ---------------T--A-------A---G-----------------T
M2    -----------------------------G--------------C-------
M3    ---------------T--A-----------G-------------------
M4    -T----------A--------------G---      T-G-------
M5    -G-----------T--------T-----------------------
M6    ---A-----A-----------------------------------
M7    -------------------------------------C-------
M10   -----------------------------------G-------
M11   --------------------------------------AA--
M12   -------------------------------------T--
M13   -------------------------------------T--
M16   ---------------TT----------------------------

DP41  TCTTGCAAGTATCACTGAGGGGGAAGAAGGAGGAGGGGCCTCCGC
B1    ----  -------A-------------------------G-------
B2    -----------------A------------------------T-G------
B5    ----------------A------------------------T-G------
B6    ----------A--A-----A-------------------------
B9    ---A-----A-----------------------------------
B10   ----------------T----------------------------
B11   ------CGCA---TTGC-A-TATC--T-----------------
B12   ---------------------------------------------
B14   ----  ----------------  ----  --  --  ----  -------
B15   ---C-----------------  ----------------  -------
```

Fig. 3 Sequence alignment of DP41 with amplified DNA products from genome of M and B plants showing differences to DP41. *Empty space* indicates a base pair deletion. For abbreviations, see Fig. 2

variable 45-bp region of the fragment (Fig. 1). However, in general more changes were observed in the Barhee sequences (specifically single base deletions) than in the Medjool sequences. Only those sequences showing differences in comparison to DP41 are shown in Fig. 3. From the 18 sequences analyzed for each variety, six of the Medjool sequences and eight of the Barhee sequences were identical to DP41. This indicates that the region of the DP41 sequence used to design the primers is common to both genomes. Among the variants found within the different genomes, two, M6 and B9, were identical. Two variants, M1 and M4, the latter with a six base deletion in its sequence, were unique to Medjool and one variant (B11) was unique to Barhee.

Primers PLM1, PLM4 and PLB11 (Table 1) were designed from M1, M4 and B11, to cover the variable portion of these sequences (Fig. 3) when used in conjunction with DP41R. All six tissue culture-derived Medjool plants and also all tissue culture-derived Barhee plants, which originated from a single mother plant, amplified a PCR product with the expected size with all three primers (data not shown). However, primer PLB11 only amplified a PCR product with the expected size of about 110 bp from two of seven non-tissue culture-derived Barhee and six of seven Medjool plants at an optimal annealing temperature of 65°C (Table 1). An identical result was observed with primer PLM1 at an optimal annealing temperature of 60°C (Table 1). Primer PLM4 (at 65°C annealing temperature), which covered a unique 6-bp deletion, amplified a PCR product from DNA of all seven non-tissue culture-derived Medjool plants (Fig. 3) but only from one Barhee plant (Table 1).
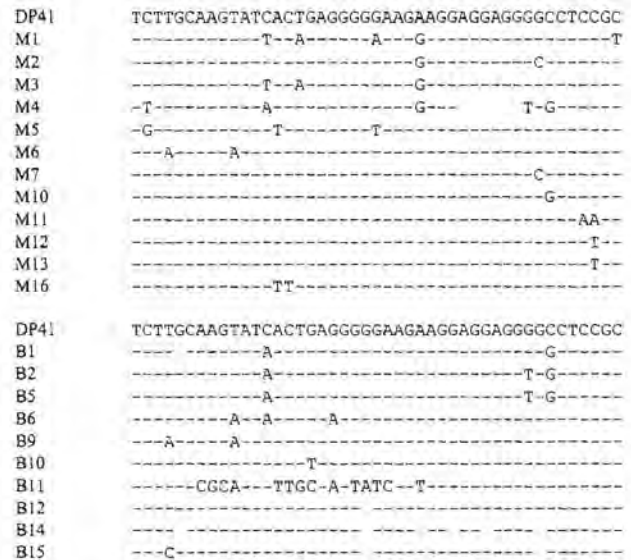
Discussion

RDA has been successfully applied in this study for date palm and has resulted in the isolation of three repetitive DNA sequences from the Barhee genome. A subset of each of these families of sequences appears either to be restricted to Barhee, or present at different multiplicity in Barhee, since the reverse subtraction with Medjool as tester yielded no products. The presence of these sequences in the DNA from both varieties could also be explained by methylation differences between the two varieties, with Medjool DNA being more methylated in this region, since *Bam*HI, a methylation-sensitive restriction enzyme, was used to generate the initial amplicons. However, the lack of difference products when *Bam*HI-derived amplicons of genomic Barhee DNA were used as a driver, or when the subtractions were performed with *Hin*dIII-derived amplicons, is consistent with a general high degree of genomic similarity between plants of the two varieties.

One sequence, which is highly variable, has been further characterized with respect to its presence in individual date palm plants of two date palm varieties. Sequence analysis of the amplified difference products from both date palm varieties identified the existence of several variant members of the repeated sequence, consistent with this family representing a variable "genetic hotspot" in the genome (Linacero et al. 2000). Variation included changes from C→T and G→A, deletion of single base pairs (which occurred at a higher frequency in Barhee) and deletion of several base pairs. Base pair changes represent the most commonly observed point mutations in plants, and can also be a consequence of plant tissue culture (Phillips et al. 1994). The extent of variation observed between plants indicates that this might be a rapidly evolv-

ing/changing sequence. We currently hypothesize that Barhee plants are generally more susceptible to these variations and that these variations have resulted in a heterogeneous Barhee population among non-tissue culture-derived plants including variations in copy number. Since all tissue culture-derived Barhee plants originated from a single Barhee mother plant, conclusions about the behavior of this family of sequences through tissue culture cannot be drawn. However, Pluhar et al. (2001) found an unequal copy number of repeated DNA among callus samples of alfalfa, and speculated that genomic stress induced by tissue culture may have caused that unequal copy number.

In the results reported here, RDA has proved to be useful in identifying a particular repetitive class of sequences that is highly variable in date palms, which is consistent with earlier observations that RDA can be used to isolate families of repetitive sequences (Cullis and Kunert 2000; Nekrutenko et al. 2000; Zoldos et al. 2001). These types of sequences are more difficult to identify with either AFLPs or RAPDs since they either result in many related polymorphisms or generate no size polymorphisms. An additional advantage of RDA is that it can also be performed using bulked amplicons, and can thus be used to identify polymorphisms that are restricted to a particular group of individuals. Therefore, bulking a series of Barhee and Medjool samples and then doing the subtraction will identify variety-specific polymorphisms, rather than individual specific polymorphisms. The types of sequences identified in these experiments would likely be identified as polymorphisms using other techniques, but due to the hyper-variability, each individual would have a unique pattern, or there would be a number of different patterns, none of which would be variety specific. The ideal probes for identifying genomes are those which have a unique location in a given genotype that can be identified directly. Application of RDA therefore offers the opportunity to generate such useful probes.

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Copy number	the number of copies of a given gene in a set of chromosomes, see multigene family.
Cross-hybridization	the binding of a probe to a DNA sequence other than the intended target sequence. This occurs because of homology between the probe and the sequence and because low stringency hybridization wash coinditions are followed.
Cultivar	a variety of plant developed through selective breeding programs.
Cytosine (C)	pyrimidine base that pairs with guanosine in DNA.
Deletion	removal of gene region or base pair from chromosome.
Denaturation	for DNA or RNA, describes separation of double-stranded molecule to a single-stranded state, usually by heating; for protein, describes change in physical shape, which is usually renders it inactive.
Digested DNA	DNA cleaved by the action of restriction enzymes.
Diploid	the normal number of chromosomes (two copies of each – 2n) in virtually all eukaryotes.
Direct repeats	multiple identical (or closely related) nucleotide sequences in the same orientation in a DNA molecule.
DNA	(deoxyribonucleic acid) the molecular basis of heredity. DNA consists of a polysugar-phosphate backbone from which the bases (nucleotides) project. DNA forms a double helix that is held together by hydrogen bonds between specific pairs of bases (thymine to adenine, guanine to cytosine). Each strand in the double helix is complementary to its partner strand in terms of its base sequence.
DNA ligase	enzyme that joins two double-stranded DNAs together, end to end, by catalyzing 3'OH and 5'P termini bond formation.
DNA polymerase	an enzyme that catalyzes synthesis of DNA under direction of a single-stranded DNA template.