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The Date Palm (*Phoenix dactylifera* L.) leaf proteome: identification of a gender biomarker to screen male parents

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Abstract

To establish a proteomic reference map of date palm leaves (Deglet Nour cultivar), we separated and identified leaf proteins using two-dimensional polyacrylamide gel electrophoresis and mass spectrometry, respectively. In total, 284 spots were excised from gel and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Among them, 158 were successfully identified (i.e, a success rate of 55.6%) conducting to the identification of 126 unique proteins. These proteins were then clustered according to their functional annotations. Identified proteins were involved in metabolism, electron transport, photosynthesis, protein synthesis, cell structure or defence. However, 29.4 % of the identifications gave unknown function. We then compared the proteome map of female and male trees. Only one discriminated spot was found to be specific of the gender. We identified the corresponding protein as an ABC superfamily ATP binding cassette transporter, ABC protein, a protein whose an ortholog in *Arabidopsis thaliana* was already reported as required for male fertility and pollen formation. The relevance of this protein as gender biomarker was then confirmed in four other cultivars, i.e., Aligue, Khouet Aligue, Kentichi and Kenta. Such biomarker should be helpful in rapidly distinguishing date palm gender of immature trees.

Keywords: Date palm; leaf proteome, gender biomarker, *Phoenix dactylifera*, sex determination.
Abbreviations: CHAPS– 3-[(3-Cholamidopropyl) dimethylammonio]propanesulfonic acid; DTT- dithiothreitol; EDTA-ethylenediaminetetraacetic acid; IEF-isoelectric focusing; PAGE- polyacrylamide gel electrophoresis; PMSF- phenylmethylsulfonyl fluoride ; PVP-polyvinylpyrrolidone; SB- sulfobetaine; TCA- trichloroacetic acid; MS/MS- tandem mass spectrometry; 2D-two-dimensional; 2-DE-two-dimensional gel electrophoresis.

Introduction

The date palm (*Phoenix dactylifera L*) plays an important role in the stabilization of ecological system in desert region where it assures the protection of interplant cropping. Its fruit constitutes the principal source of income economical for people living in oasis. Few genomic studies on date palm tree have been performed, however. Recently, Al-Dous et al. (2011) published a first draft of the date palm tree genome: genome annotation allowed the prediction of more than 25,000 genes (http://qatar-weill.cornell.edu/research/datepalmGenome/download.html).

A limited number of date palm proteomic investigations have been published up to now (Gómez-Vidal et al., 2008; Sghaier-Hammami et al., 2009a; Sghaier-Hammami et al., 2009b, Gómez-Vidal et al., 2009). An immune-proteomics approach allowed the identification of 2 new allergens in pollen of date (Postigo et al., 2009). More recently, the characterisation of proteins contained in juice tapped from Deglet Nour variety established a proteomic map of sap (Ben Thabet et al., 2010). In the present study, we give the first proteomic map of date palm (Douglet Nour cultivar) leaves. Identified proteins were grouped according to their putative function. The date palm being dioecious, with separate male and female trees (Ainsworth et al., 1998), we then compared the proteomic maps according to the tree gender. Indeed, the inability to distinguish between male and female trees at an early stage is yet a major problem for date palm biotechnology. Therefore the ability to use genetics to predict the gender of immature trees is one of the most immediate challenges of date palm biotechnology. In their paper, Al-Dous et al. (2011) began to dissect genomic differences between male and female trees. Scaffolds which they identified to be linked to gender form the basis of DNA marker-based gender test. Investigation of these regions showed that the date palm uses a XX/XY sex determination system with the male being the heterogametic sex. The present study allowed to discriminate a protein that is

specifically accumulated by male trees. The validity of this polypeptide as sex-marker was then confirmed in Aligue, Khouet Aligue, Kentichi and Kenta tree varieties.

Results and Discussion

Proteomic map of the Deglet Nour Palm tree leaf

A representative image of the 2-DE gel obtained from the date palm leaf (Deglet Nour cultivar) is presented in Fig.1. A total of 284 protein spots were discriminated, excised from the 2D-PAGE gels and then digested. Lastly, 158 were successfully identified conducting to 126 unique polypeptides. Results are listed in supplementary data in which identified proteins were clustered according to their functional annotation following criteria of Bevan et al. (1998). The identification efficiency which we obtained (55.6%) is close to that reported for a proteomic study performed on wheat leaves, in which 51% of proteins were identified (Donnelly et al., 2005). The gap between the number of identifications and that of proteins is explained by the presence of multiple spots for some single proteins. This phenomenon is commonly found on 2D gels because the migration of proteins on a 2D-PAGE gel is very sensitive to small structural differences (Xu et al., 2006; Sarnighausen et al., 2004; Giavalisco et al., 2005). In this study, 22 proteins exhibited multiple spots, e.g., the heat shock protein 70 (spots 1–4), the rubisco large subunits (spots 27, 28, and 277), the triose-phosphate isomerase (spots 181, 182, 200, 223 and 229). Three major reasons may be advanced for this phenomenon. These spots might correspond to isoforms derived from different genes of a multigene family (Xu et al., 2006), the date palm genome being expected to contain multiple copies of many genes. The distinct biophysical properties that govern their migration behaviour in the two dimensions are therefore might be due to differences in the amino acid sequences. It is also possible that one gene product may undergo different co- and/or posttranslational modifications that affect protein mass or/and charge (Sarnighausen et al., 2004). The appearance of multiple spots corresponding to one gene product is a consequence of artificial modification of proteins, such as carbamylation, during the extraction or separation procedure (Berven et al., 2003). However, it is improbable that the multiple spots in this study are artefacts of protein damage during sample preparation because we used appropriate precautions for prevent artificial modifications and the multiple spots are highly reproducible. Data show that 7.1% of the identified proteins are orthologs of *Arabidopsis* genus polypeptides. This observation agrees well with recent data which showed that oil palm and date palm transcript sequences closely matched with *Arabidopsis* orthologs, because it is far the best-annotated proteome among the plant kingdom (Bourgis et al., 2011). Only one spot (71) was identified from the *Phoenix dactylifera* genome. This observation, quite surprising, might be explained by the poor representation of the date palm tree species in databases (448 protein entries for July 2012 at NCBI). Most of the identified proteins (35.7%) were involved in metabolism as would be expected in plant leaf tissue (Porubleva et al., 2001; Donelly et al., 2005). These proteins include those involved in glycolysis, gluconeogenesis, pentose phosphate pathway, TCA cycle, respiration, fermentation, electron transport, and photosynthesis. Some proteins playing a role in photosynthetic electronic transport were identified, i.e., PS I subunits (spots 183, 185 and 266) PS II subunit (spots 152 and 222); thylakoid lumenal 17.4 kDa protein (spot 92); thylakoid lumenal 19 kDa protein (spots 160 and 161) and NADH-dependent hydroxypyruvate reductase (spot 270). Several enzymes of the carbon metabolism were also identified, e.g., rubisco activase, rubisco large subunit, malate dehydrogenase (spot 262), sedoheptulose-1.7-biphosphatase (spots 78 and 79), phosphoglycerate kinase (spots 55, 56, 62 and 65), glyceraldehyde-3-phosphate dehydrogenase (spot 271), triosephosphate isomerase (spots 181, 182, 200, 223 and 229) and transketolase (spot 11). Rubisco is the primary enzyme in photosynthetic carbon fixation and the likely rate-limiting factor for photosynthesis under light-saturated conditions (Makino et al. 1985). The ATPase is composed of two structural domains: a hydrophobic membrane-bound portion called CF_0, and a soluble portion that sticks out into the stroma called CF_1. CF_1 consists of 5 different subunits: α-, β-, γ-, δ-, and ε-units (Xu et al., 2006; Blankenship, 2002). Only the soluble subunit β (spots 46, 47, 49, 51 and 71) of the ATPase complex was found on the 2D-PAGE gels. Polypeptides classified as disease and defence-related proteins (16.6% of the identified proteins) include resistance proteins, defence regulated proteins, those involved with cell death, cell rescue, stress responses, and detoxification (Bevan et al., 1998). Among these proteins were superoxide dismutases (spots 118, 149, 158, 159, 182 and 244) and the peroxiredoxin (spots 96, 167 and 168) which are involved in the response to oxidative stress. Two heat shock proteins (HSPs) were identified, i.e., the HSP 70 (spots 1, 2, 3, 4) and the 18 kDa HSP (spot 146). HSPs are associated with protein folding, protein translocation across membranes, assembly of oligomeric proteins, modulation of receptor activities, mRNA protection, prevention of enzyme denaturation and their stress-induced aggregation, and with post-stress ubiquitin and chaperonin-aided repair. Based on these functions, HSPs have been termed "molecular chaperones" (Xu et al., 2006).
Thirty-seven unclassified proteins were grouped in unknown function, predicted protein and hypothetical protein. The percentage of these proteins (29.4%) is here higher than those reported by Donelli et al. (2005) and Watson et al. (2003) but lower than that given by Porubleva et al. (2001).

Comparison of the proteomic maps of male and female trees

One of the challenges encountered by the date palm biotechnology is the inability to distinguish female from male trees at an early stage. Date palms take 5-8 years after planting to flower, the earliest point at which male and female trees can be distinguished today. Consequently, distinguishing the tree gender will be of a great economical interest. It is the reason why many groups have attempted to find sex-linked markers in date palm to assist its development (Bekheet and Hafany, 2011). Some have identified DNA-markers that segregate with sex in one or two varieties (Younis et al., 2008), but to date, none have found markers working across a broad set of dare palm cultivars. Recently, Al-Dous et al. (2011) identified linked regions in the date palm genome that were linked to gender. The same group, more recently, developed PCR-based assays capable of sex differentiation in multiple date palm cultivars (Al-Mahmoud et al., 2012). However, none protein biomarker of the date palm tree gender has been identified up to now. We consequently decided to use the high resolution of proteomics to compare the proteomes of male and female trees in order to find such marker. Results (Fig. 2A) showed very close proteomes in male and female trees. Indeed, only one spot (circled spot on Figure 2A) was found as sex-dependent, since only present in male. This spot was not initially found on the whole proteome (Fig 1), obtained from female trees

Table 1. Mass spectrometry data of the gender-specific protein.

Spot	Peptide sequence	Protein name	Organism	NCBInr Accession number	MW/pI		Score	Coverage %
					Theoritical	Estimated		
95	GAGKTTLVKH	ABC superfamily ATP binding cassette transporter, ABC protein	*Corynebacterium glucuronolyticum*	gi|227487557	23/5.90	11/5.70	67	37.31

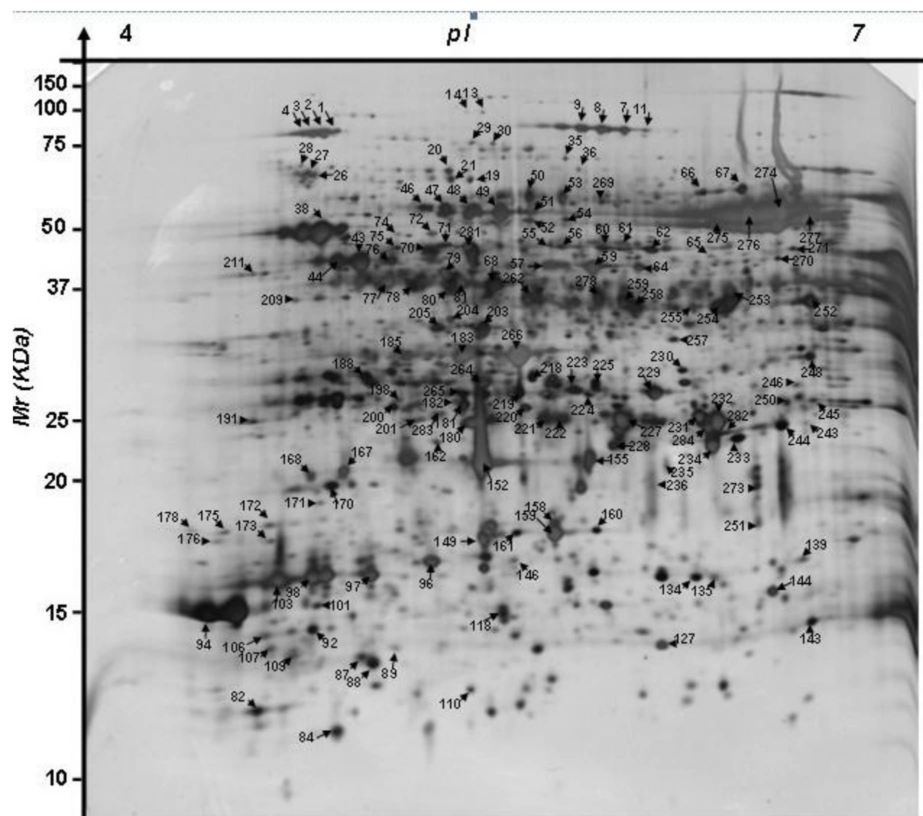

Fig 1. Silver stained 2D-PAGE gel of date palm leaf proteins. Proteins were separated in the first dimension on an IPG strip pH 4.0–7.0 and in the second dimension on a 13.75% acrylamide SDS-gel. The numbered spots were identified and are presented in Table 1.

which are in majority in palm groves. The gender-specificity of this spot was then confirmed in four other cultivars, i.e., Aligue, Khouet Aligue, Kentichi and Kenta. (Fig. 2B)

The ABC protein and its application as biomarker to detect gender in date palm

The protein corresponding to this gender specific spot was identified as belonging to the ABC superfamily ATP binding cassette transporter (Table 1). A comparison of theoretical and experimental protein masses shows that the predicted size of the protein is much higher than the observed size, probably due to a degradation of the protein. This well conserved protein family translocates a wide range of solutes across extra- and intracellular membranes and is represented in almost all known organisms from bacteria to human. In *Arabidopsis thaliana*, one of the ABC super family ATP binding cassette transporters has been recently shown as required for normal male fertility and pollen development (Guilichini et al., 2010). The corresponding gene was tightly co-expressed with genes required for sporopollerin biosynthesis and highly expressed in the early flower bud, supporting a role in pollen wall formation. A mutation on this gene induced a strong reduction of fertility. These observations accord well with a male specificity of this protein. Such marker might be very useful to improve the organization of palm groves where the ratio of male to female usually is kept at between 1:25 and 1:50 whereas reproduction leads to a population composed of male and female in equal proportion (50% each). In this context, the ability to quickly replant orchards from seeds or seedlings known to be female would be of great benefit.

Materials and methods

Plant material

Leaves from mature date palm leaves were collected from arid south Tunisia (Degache town) in May 2009. Samples were frozen in liquid nitrogen and stored at (-80°C) for later analysis. Deglet Nour, Kentichi, Aligue, Kenta and Kouet Aligue cultivars were used

Protein extraction

5 g of frozen leaves were ground in a mortar with liquid nitrogen. The leaves powder was then suspended in 16 ml of extraction buffer (containing 0.7 M saccharose, 0.5 M Tris,

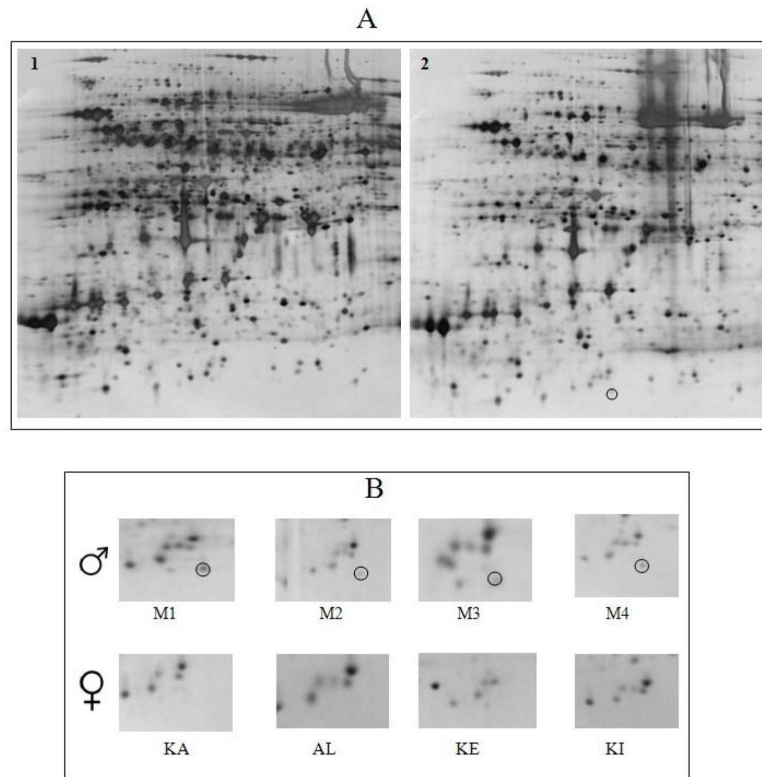

Fig 2. Comparison of the proteomic maps of male and female trees. A: Comparison of the proteomes of female (1) and male (2) Deglet Nour trees. B. Magnification views of the proteomic maps of four male trees (M1, M2, M3 and M4) with those of female trees from 4 cultivars: Khouet Aligue (KA), Aligue (AL), Kenta (KE) and Kentichi (KI). The spot specifically present in male trees is circled

0.1 M KCl, 2% ß-mercaptoethanol, 50 mM EDTA, 2 mM PMSF, adjusted pH to 7.5 by HCl) and 25% (w/v) PVP. This suspension was shaken for 10 min at room temperature and then centrifuged (20 min, 1,789 x g, 4°C). The supernatant was centrifuged again at 10,000 x g for 20 min at 4°C. Afterward, an equal volume of water-saturated phenol was added. Samples were shaken for 10 min at room temperature and then centrifuged (20 min, 13,000 x g, at room temperature) to separate the phenolic and aqueous phase. The phenolic phase was recovered and extracted again with the same volume of extraction buffer. Subsequently, centrifugation was repeated and 5 volumes of precipitation solution (0.1 M ammonium acetate in methanol) were added to the phenol phase. Proteins were precipitated at -20°C overnight. After centrifugation (for 10 min, 15,700 x g, 4°C), the protein pellet was washed three times with the precipitation solution. Each washing step was followed by a 5 min centrifugation (Schuster and Davies 1983). After drying under vacuum, the pellet was resuspended in 15 % (w/v) TCA and incubated for 3 hours on ice. After centrifugation (for 15 min, 15,700 x g, at 4°C), the protein pellet was washed three times with cold acetone. Each washing step was followed by 5 min of centrifugation as described above. The pellet was solubilized in IEF buffer containing 8 M urea, 4% (w/v) CHAPS, 10 mM DTT and 2% carrier ampholytes pH 3-10 (Amersham Biosciences).

Two-dimensional gel electrophoresis

For all experiments, protein concentrations were evaluated using the Bio-Rad Protein Assay. For the first dimension (IEF), 94 µg of proteins were solubilised in IEF buffer (final volume 300 µL) of the following composition: Urea, 5M; thiourea, 2 M; CHAPS, 2%; SB(3-10), 2%; Tris, 40 mM; Bio-lyte carrier ampholytes (pH 3.5-10) from Bio-Rad, Hercules, CA, USA . The first-dimensional gel separation was carried out with Immobiline Dry Strips L (18 cm, pH 4-7, Amersham Pharmacia Biotech, Uppsala, Sweden). IEF was performed with the IEF-CELL (Bio-Rad) as follows: active rehydratation for 10 h at 50 V, 250 V for 15 min, gradient from 250 to 10,000 V for 3 h and final focusing for 12 h 15 min at 10,000 V. The strips containing focused proteins were then stored at -20°C. After IEF, strips were equilibrated in a buffer containing 1% DTT for 10 min. A second equilibration step was performed for 10 min in an equilibration buffer containing 4% iodoacetamide. Lastly, the second dimension was ensured by sodium dodecyl sulphate polyacrylamide gel electrophoresis using a 13.75 % polyacrylamide resolving gel (width 16 cm, length 20 cm, thickness 0.75 cm). Experiments were carried out using the protean II Xi vertical systems (Bio-Rad) (Khemiri et al. 2008). After migration, proteins were visualized by silver nitrate staining (developing duration: 17 min) as described by Rabilloud et al. (1994). Three analytical replicates gels were analyzed per sample, and scanned by computing scanning densitometry (ProXpress; PerkinElmer). Images were then imported into the image analysis software Progenesis SameSpots v3.0 (Nonlinear Dynamics).

Gel analysis and protein identification

Spots were excised from the polyacrylamide gel with an automatic spot cutter (ProXCISION, PerkinElmer). To elaborate the proteomic map of the *Deglet Nour* palm tree leaf, all discriminated spots were excised. To compare male and female proteomes, three 2-D gels were matched together

to form a reference image. The protein spot volumes were automatically normalised in the software. A threshold of ANOVA (p) < 0.05, $q < 0.05$, power >0.8 and at least two-fold change in average spot volume was used to define the protein spots which differ according to the gender. In this case, only spots fulfilling these criteria were excised. Gel plugs were then dried using a SpeedVac centrifuge for a few minutes. Trypsin digestion was performed using an automatic digester (MultiPROBE II, PerkinElmer). After lyophilization, the peptide extracts were resuspended in 10 µL of 0.1% formic acid/3% acetonitrile. Peptides were enriched and separated using a lab-on-a-chip technology (Agilent, Massy, France) and fragmented using an on-line XCT mass spectrometer (Agilent) according to manufacturer. The fragmentation data were interpreted using the Data Analysis software (version 3.4, Bruker Daltonic, Billerica, MA, USA). For protein identification, MS/MS peak lists were extracted and compared to the NCBInr protein database restricted to the green plants *(Viridiplantae)*, using the MASCOT Daemon (version 2.1.3) search engine. All searches were performed with no fixed modification and allowed for carbamidomethylation, oxidation and a maximum of one missed trypsin cleavage. MS/MS spectra were searched with a mass tolerance of 1.6 Da for precursor ions and 0.8 Da for fragment ions, respectively. If a protein was characterized by 2 peptides with a fragmentation profile score higher than 53 (default value of MASCOT), the protein was validated. When one of the criteria was not met, peptides were systematically checked and/or interpreted manually to confirm or cancel the MASCOT suggestion.

Conclusion

We gave here first the date palm proteome reference map. We then compared the proteomes of male and female trees. These assays allowed identifying a protein which segregates with sex and works across three cultivars. This is for our knowledge the first protein gender-biomarker identified up to now for this species. Such tool may be very useful for date palm biotechnology, this crop being critical to agriculture in many hot and arid regions**.**

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In memory of Dr Mokhtar Trifi who has left us in 2010.

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