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Identification and characterization of differentially expressed ESTs in date palm leaves affected by brittle leaf disease

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ABSTRACT

Brittle leaf disease (BLD) of date palms was first described in the south of Tunisia in 1960. Since then, it progressively spread out to reach epidemic proportions in 1986. Both biotic and abiotic factors have been suggested to be possible causal agents of this disease. However, the research efforts aimed at identifying the causal agent of this disease have still been unsuccessful. In order to get an insight in the molecular mechanisms involved in BLD of date palm, we have constructed suppression subtractive hybridization libraries from affected (ABLD) and healthy leaves (HBLD). This report describes the cloning, sequencing and characterization of 74 independent cDNA derived from the ABLD and the HBLD subtractive libraries. BLAST analysis showed that the ESTs from HBLD library were mainly related to photosynthesis, protein synthesis and ion transport whereas the EST sequences from ABLD were related to stress responsive genes, metabolism associated, protein synthesis and signal transduction.

1. Introduction

Date palm (*Phoenix dactylifera* L.) is one of the oldest fruit crops grown in the arid regions of the Arabian Peninsula, North Africa, and the Middle East. The most probable area of origin of the date palm was in or near what is now the Iraq country, but date cultivation spread to many countries started in ancient times. Dates are a major food and income source for local populations in the Middle East and North Africa, and play significant roles in the economy, society, and environment in these areas [1]. In Tunisia, date orchards are being decimated by a new disease called brittle leaf disease (BLD) of unknown origin [2]. The disease was observed on most Tunisian varieties including the elite one, *i.e.* Deglet Nour as well as on seedling trees and pollinator ones. The disease evolved in three stages (Fig. 1). In the first stage (S1), few fronds become chlorotic with a dull, olive green color (Fig. 1B). Later on, in the second stage (S2) leaflets become brittle, twisted, frizzled and shriveled with a scorched appearance (Fig. 1A). The most characteristic symptom is the ease with which leaflets can be broken. Necrotic streaks develop then on the pinnae at the second stage. In the third stage (S3), these symptoms gradually extend to the nearby fronds until the whole tree is affected, and dies (Fig. 1A). Four to six years may elapse between first symptom appearance and death of the tree. Symptoms occur on trees of all ages, including offshoots and small seedlings.

Mineral analysis has shown that adult leaflets from BLD-affected palm trees are severely deficient in manganese, suggesting that BLD is a nutritional disorder [2,3]. However, other observations suggested horizontal transmission from an initially affected tree to neighboring ones, indicating a clustering and a non-random spread. Such a pattern is rather characteristic of biotic diseases, although no pathogen has been found yet [2]. Recently, Namsi et al. [4] established a molecular diagnosis method based on the detection of BLD associated chloroplast encoded double stranded RNAs; they also reported that brittle leaf disease may be due to mineral elements deficiency and does not involve any pathogen [3].

Over the recent years, plant sciences have advanced with abilities and capabilities allowing molecular level studies towards elucidation of disease susceptibility and resistance mechanisms. Transcriptomic studies are efficient approaches for the identification of genes involved in response to biotic and abiotic stresses in plants. The suppression subtractive hybridization technique

Abbreviations: BLD, brittle leaf disease; EST, expressed sequence tag; MT, metallothionein; HBLD, reverse suppression subtractive hybridization; RT-PCR, reverse transcription-PCR; ABLD, forward suppression subtractive hybridization; SSH, suppression subtractive hybridization.

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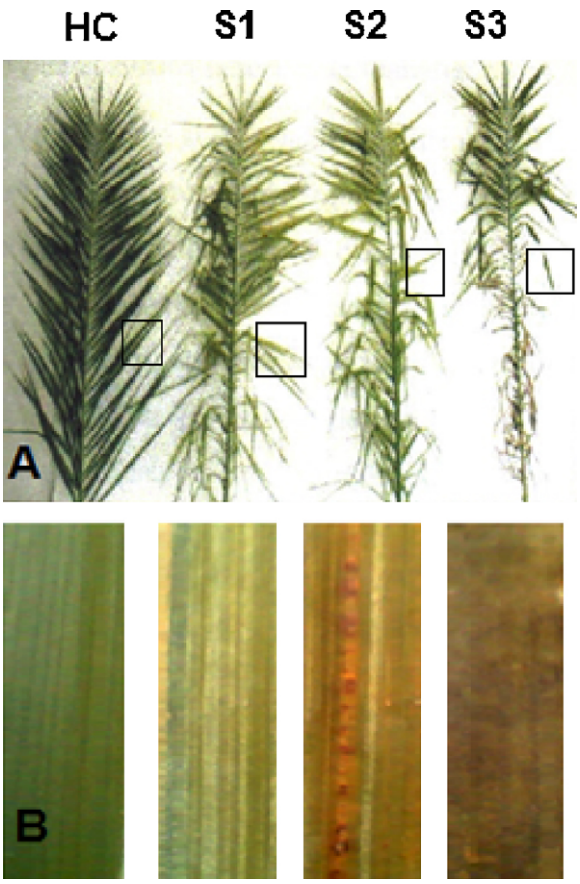

Fig. 1. (A) General view of affected frond. (B) Detailed view of representative leaflets. HC: healthy control leaf. S1: Stage 1, corresponding to early chlorotic symptoms on leaves; S2: Stage 2 with brittle leaves with chlorosis propagation and brown color; S3: Stage 3, latest stage of the disease; leaves become dried and totally necrotic.

(SSH) which allows the identification of genes that vary in their expression levels during different biological processes, has been successfully used in higher eukaryotes to study the molecular response to different diseases [5].

Although a draft of the date palm genome has been made public recently (http://qatar-weill.cornell.edu/), no transcriptomic studies have been reported so far in the literature for this tree species of great economical, environmental and social importance in the Middle East and North Africa countries. It is therefore of importance to elucidate the molecular pathways occurring in plants affected by brittle leaf disease to enable discovering the processes involved in the disease and to better understand the causal agents.

As a first step towards understanding the molecular events underlying BLD, we used SSH to construct subtractive cDNA libraries between healthy and affected date palm leaves. The differentially expressed cDNA fragments were sequenced and analyzed against NCBI databases in order to identify their putative function. We also used semi-quantitative RT-PCR to study the transcript accumulation of a subset of these genes in leaves harvested at three stages of the disease.

2. Materials and methods

2.1. Plant material

Healthy control and BLD-affected date palm leaves from different stages of the disease were collected from south Tunisian oases (Degache) frozen in liquid nitrogen and stored at −80 °C until use. The typical three stages S1, S2 and S3 of BLD were identified on the basis of symptoms exhibited by leaves (see Fig. 1).

2.2. RNA isolation

Leaf tissues were ground in liquid nitrogen and total RNA was isolated as described previously [6]. A "forward" SSH cDNA library (affected versus healthy leaves, ABLD), was constructed by using as the driver, the cDNA from affected leaves, at the second stage of the disease (S2), which was subtracted from the tester cDNA produced from healthy leaves of a non-affected tree. In the "Reverse" SSH cDNA library (healthy versus affected leaves HBLD), the driver cDNA (healthy leaves) was subtracted from the tester cDNA (affected leaves). Both libraries were constructed using the Clontech PCR-Select^{TM} cDNA Subtraction Kit according to the manufacturer's instructions. Amplified, differentially expressed cDNA fragments were cloned in a pGEM-Teasy vector (Promega) and transferred to ultra competent DH5α cells (NEB).

2.3. Screening of the SSH library

Differential screening was carried out by dot blot hybridization. About 3 μl of the PCR product, amplified from each of the 1056 individual clones with universal M13 sequencing primers, were mixed with 3 μl of 0.6N NaOH for denaturing, and then 3 μl of each of denatured PCR product was arrayed onto two identical nylon membranes. After air-drying, membranes were UV-cross-linked. Biotin-labeled cDNA probes were synthesized from the tester as well as the driver cDNAs by PCR using SMART cDNA synthesis kit (Clontech). They were purified using the PureLink PCR purification Kit (Invitrogen). Two sets of membranes were hybridized with Biotin-labeled driver or tester cDNAs. The hybridization was carried out at 42 °C overnight in the prehybridization/hybridization buffer (5× SSC, 1× Denhardt's solution, 25 mM sodium phosphate [pH 6.5], 45% formamide, and 0.2 mg/ml freshly denatured salmon sperm DNA). The membranes were washed with 2× SSC, 0.2% (w/v) SDS at 42 °C for 10 min, and then with 0.1× SSC, 0.1% SDS at 42 °C for 10 min. Blots were detected using Immunstar HRP substrate (Biorad) and the membranes were exposed to X-ray film for signal detection.

2.4. Sequence processing and analysis

Based on the results of dot blot hybridization, 298 differentially expressed clones were selected and sequenced, raw files were manually processed using BioEdit software to remove the vector sequences and the polyA tails. Each individual edited EST was queried to the non-redundant NCBI (http://www.ncbi.nlm.nih.gov) databases with a significant cut-off value of 1e−5 using BLASTX search to assign a putative function. Sequences that did not show any significant homology were searched again using BLASTN against sequences from a draft of the date palm genome (ACYX00000000.1) and putative function were determined from available annotated sequence package (http://qatar-weill.cornell.edu/). All EST sequences were submitted to the EST division of GenBank NCBI (http://www.ncbi.nlm.nih.gov/dbEST/), under the following numbers presented in Tables 1 and 2 for up-regulated ESTs and down-regulated ESTs, respectively.

2.5. Semi-quantitative analysis of gene expression by RT- PCR

Semi-quantitative determination of the level of differentially expressed transcripts was carried out as described [7], RT-PCR were performed in triplicate. Single stranded-cDNAs were synthesized from 2 μg of total RNA of healthy and diseased leaves, taken at the different stages using 200 units of MMLV reverse transcriptase