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ORIGINAL PAPER

Identification and characterization of drought stress responsive genes in faba bean (*Vicia faba* L.) by suppression subtractive hybridization

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Abstract Faba bean (*Vicia faba* L.) ranks fourth in food legume crop production in the world. However, drought is a potential major constraint to faba bean production and improved faba bean cultivars and development of drought-resistant varieties play a key role in enhancing faba bean crop production. In this study, suppression subtractive hybridization (SSH) technique was used to study differential expression in response to water stress and to identify genes involved in molecular mechanism of drought tolerance. A forward subtractive cDNA library induced by water deficit conditions was constructed used Hara faba bean cultivar grown in pots and treated with either well-watered (WW) or water-stressed (WS). A total of 28 clones were identified as drought stress induced. After sequencing, ten unique expressed sequence tags (ESTs) were obtained by clustering and blast analysis which showed homology to known drought responsive genes including heat shock protein (*HSP*), late embryogenic abundant (*LEA*), zinc finger protein transcription factors (*ZFP*), lipid transfer protein (*LTP*), chlorophyll a/b-binding protein (*ChlBP*), thioredoxin h (*Trx h*), and *ATP synthase* as well as some functionally unknown transcripts. Their expression was characterized in Leaf, root, flower, cotyledon, and stem tissue. Quantitative RT-PCR analysis revealed that eight genes were consistently up-regulated in Hara compared to Giza3 cultivar, known as drought-tolerant and sensitive respectively under water deficit treatment. The expression of six genes was differentially expressed in different stages of water stress faba bean plant. Drought responsive genes showed changed expression patterns, indicating that they may play important roles in faba bean water stress response. Furthermore, these results indicate that drought-induced genes are related to metabolic pathways and genetic regulation of stress and development and can serve as a foundation for future studies to elucidate drought stress mechanisms of faba bean.

Keywords Drought stress · Faba bean (*Vicia faba* L.) · Gene expression · Suppression subtractive hybridization (SSH)

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Introduction

Faba bean (*Vicia faba* L.), is among the oldest crops in the world and the most widely grown protein-producing food legumes. In developing and industrialized countries faba bean is used as human food and animal feed respectively (Mulualem et al. 2012). Moreover, faba bean is a suitable

legume crop improves soil fertility by fixing atmospheric nitrogen and increase yields of succeeding. The world faba bean area harvested in 2012 stands at 2.56 million ha (FAOSTAT 2012). In Tunisia, faba bean is one of the most grown and consumed food legumes and is grown mostly in the north of the country where rainfalls reach more than 400 mm per year in average (Kharrat and Ouchari 2011). The total acreage of faba bean for dry seed harvesting in 2010 was 58.800 ha and represented about 68 % of total grain legume area in Tunisia (Kharrat and Ouchari 2011).

The average world grain yield was 1.7 t/ha in the period of 2008–2010 (FAOSTAT 2012). In Tunisia, recent data showed that average yields were 0.99 and 0.76 t/ha for faba bean small and large seed respectively (Kharrat and Ouchari 2011). Indeed, the national average yield of faba bean is low and varies tremendously from year to year. This fluctuation is due to many factors such as the lack of improved cultivars, the high susceptibility of commercial varieties to diseases and pests and the sensitivity of the crop to environmental conditions (Kharrat et al. 2006). The most important abiotic stress in Tunisia is drought (Rejili et al. 2008). Moreover, Link et al. (1999) suggested that low yield potential in faba bean is partly caused by drought susceptibility. Furthermore, drought reduces average yields in faba bean at different stage of plant development, particularly at early podding stage which showed reduction in faba bean yield by 50 % (Mwanamwenge et al. 1999).

Drought stress remains the most important environmental factors inhibiting photosynthesis, altered hormonal balance and plant mineral nutrition and decreasing growth and productivity of plants (Zlatev and Lidon 2012; Gholami et al. 2013; Khodadadi 2013; Tozzi et al. 2013). Plants respond to drought stress through a number of biochemical, physiological, molecular and developmental changes (Atkinson et al. 2013). Drought tolerance is a complex trait that is influenced by environmental interactions and controlled by multiple genes (Pinto et al. 2010). Furthermore different species and cultivars of crops show variation in their drought tolerance, hence the importance of genetic diversity as an underlying factor of drought tolerance (Budak et al. 2013). Indeed, use plants exhibiting drought tolerance is the suitable approach for breeding of new varieties more tolerant to drought stress (Onemli and Gucer 2010).

In many plant species, including barley, maize, rice, and Arabidopsis several genes with various functions related to drought tolerance have been drawn out, identified and characterized, but little is known about the relationships of these genes under drought stress (Atkinson et al. 2013; Nguyen and Sticklen 2013; Sahoo et al. 2013; Van Houtte et al. 2013). Endogenous abscisic acid (ABA) levels have been reported to increase as a result of water deficit in many physiological studies, and therefore ABA is thought to be involved in the signal transduction. ABA plays an important role in the stress response and tolerance of plants to drought and high salinity (Marcińska et al. 2013; Nakai et al. 2013; Okamoto et al. 2013). ABA response pathway acts through a complex signaling cascade induces nucleotide-binding proteins, protein degradation pathways, secondary messengers, protein kinase/phosphatase cascades, and transcription factors (Himmelbach et al. 2003; Lindemose et al. 2013). In general, target genes containing the *DRE* (dehydration-responsive element) or *DRE*-related core motifs (CCGAC) in their promoter regions are induced in response to drought conditions (Shinozaki and Yamaguchi-Shinozaki 2007). Among these genes, *rd29A* (cor78/lti78) encodes a protein similar to *LEAs* (late embryogenesis abundant proteins) that was able to protect cellular structures in *Arabidopsis thaliana* under water stress conditions (Shinozaki and Yamaguchi-Shinozaki 2007). Moreover, *CBF* (C-repeat binding factor) genes, also known as *DREB* (dehydration responsive element binding) genes encode a transcription factors (TFs) regulating stress-inducible gene expression. Over-expression of *GmDREB2*, a soybean DRE-binding transcription factor gene showed enhanced plant tolerance to drought and high-salt stresses (Chen et al. 2006). In the other hand, the over-expression of *LsDREB2* increased the tolerance of transgenic lettuce (*Lactuca sativa* L.) only to salt stress (Kudo et al. 2014). Transcriptome analysis was used to identify candidate genes for drought tolerant and characterization of the plant response to various abiotic stresses in many plants, including hyacinth bean (Yao et al. 2013) and maize (Shan et al. 2013).

Breeding strategies and genetic engineering have been reported in order to incorporate genes associated with water stress tolerance in major food crops. In faba bean, there are few reports about genes conferring drought tolerance compared to biotic stress. Breeding has been used to improve the drought tolerance of faba bean, but so far the progress with this approach has been slow and limited (Gnanasambandam et al. 2012). Information about genes involved in faba bean drought stress should be helpful and provide tools to develop drought tolerant faba bean cultivars.

Several techniques have been used to identify the genes expressed in response to drought stress, including differential display (Rahman et al. 2013; Soni et al. 2013), cDNA-AFLP (Gupta et al. 2013; Pareek et al. 2013), microarrays technology (Bhargava et al. 2013; Liu et al. 2013b), serial analysis of gene expression (Cheng et al. 2013b), massively parallel signature sequencing (Reinartz et al. 2002) and suppression subtractive hybridization (Yao et al. 2013). Suppression subtractive hybridization (SSH) method is a powerful approach and a valuable tool for identifying differentially regulated genes important for cellular growth and differentiation and for abiotic/biotic

response (Garg et al. 2013; Luo et al. 2013). SSH appears to produce less false positives compared to other methods (Diatchenko et al. 1999). Accordingly, SSH should facilitate the identification of low-abundance, differentially expressed genes involved in faba bean drought response.

This study reports the use of SSH to construct a subtractive cDNA library of drought-stressed faba bean and the identification of drought-induced expressed sequence tags (ESTs). These genes may be candidates for molecular markers, which could assist the domestication and selective breeding programs of faba bean.

Materials and methods

Plant materials

Faba bean (*Vicia faba* L.) cultivars Hara (cultivated in semi-arid climate regions of Tunisia) and Giza 3 with sensitivity to drought stress (Abdellatif et al. 2012) were used in this study. Morphological characteristics from ten plants per cultivar were recorded using five descriptors (Table 1) including qualitative characters (coat colour and flower colour) and quantitative plant and seed traits (1,000-seeds weight, plant height and days to beginning of flowering) chosen from faba bean descriptors of the International Board for Plant Genetic Resources (IBPGR 1985).

Seeds were surface sterilized using 5 % sodium hypochlorite solution for 5 min and then rinsed three times with sterile distilled water. The seeds were then soaked overnight in sterile distilled water. Then, the faba bean seeds were sown in plastic pots filled with 5 kg of air-dried soil.

For SSH library construction, ten faba bean plants (Hara cultivar) were grown in the greenhouse of the Experimental Station (Center of Biotechnology of Borj Cedria) under cycles of 16/8 h day/night photoperiod and at a controlled temperature of 25 °C. Pots were divided into two groups. The control pots were well watered (WW) every day, whereas the water stress (WS) treatment was applied at the four true-leaf growth stage (15 days after sown). After 10 days under drought treatment (25 days after sown), leaf tissues from WW and WS plants were harvested, frozen immediately in liquid nitrogen and stored at −80 °C.

RNA isolation

For each sample 0.2 g of leaf tissue was ground into a fine powder in liquid nitrogen with a mortar and total RNAs were extracted following the protocol described by Chang et al. (1993). Poly $(A)^+$ RNA was subsequently purified using an Oligotex™ mRNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's guidelines.

Construction of suppression subtractive hybridization library

Subtractive hybridization was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) according to manufacturer's instructions. In brief, first and second strand cDNA were synthesized from 2 µg of poly $(A)^+$ RNA from the driver (Hara cultivar under well-water conditions) and the tester (Hara cultivar under water-stressed conditions) tissue. Tester and driver cDNA were digested with *Rsa*I and purified using phenol/chloroform/isoamyl alcohol mixture (25:24:1). Digested cDNA was electrophoresed on a 1 % agarose gel and stained with ethidium bromide. Adaptors 1 and 2 from the PCR-Select cDNA subtraction kit were ligated to tester cDNA. To assess ligation efficiency, a PCR amplification test was performed according to the protocol in the PCR-Select™ cDNA subtraction kit. The first and second hybridizations were performed according to the PCR-Select cDNA subtraction kit protocol. Product from the final hybridization was then diluted in 200 µl of dilution buffer (20 mM HEPES pH 8.3, 50 mM NaCl and 0.2 mM EDTA) and heated at 68 °C for an additional 7 min. After the primary and secondary hybridization, two successive PCR amplifications were performed. The 25 µl PCR amplification mixture contained 1 U Taq DNA polymerase (Thermo Fisher Scientific, Erembodegem, Belgium), 2.5 µl of 10× PCR buffer, 100 µM dNTP, 2.5 mM $MgCl_2$ and 1 µl of each primer (10 µM). The first PCR was carried out using 1 µl diluted hybridization mixture and the target sequences were amplified using P1 and P2 primers with the following parameters in a MyCycler™ thermocycler (Bio-Rad. Laboratories, Foster City, CA, USA): 5 min initial elongation at 72 °C, 30 cycles of 30 s at 94 °C, 68 °C for

Table 1 Origin and estimation of some traits of faba bean (*Vicia faba* L.) cultivars

Cultivar	Country of origin	Plant height (cm)	Days to beginning of flowering	Flower colour	Seed characteristics	
					Coat colour	TSW (g)
Hara	Tunisia	$81.93^a \pm 4.82$	$58.13^a \pm 2.68$	White	Brown	$923.60^a \pm 18.61$
Giza 3	Egypt	$84.26^a \pm 3.95$	$50.33^b \pm 1.15$	White	Brown ochre	$954.70^a \pm 20.30$

[a,b] Homogenous groups following the Newman and Keuls test

30 s, 72 °C for 1.5 min followed by a final extension at 72 °C for 5 min. One microlitre of the first PCR products was used as a template in secondary PCR using the nested PCR primer PN1 and PN2. PCR was performed for 15 cycles (94 °C for 30 s, 66 °C for 30 s, 72 °C for 1.5 min). For the evaluation of subtraction efficiency, the faba bean specific elongation factor primers were designed (Table 2). After subtraction, the secondary PCR products were purified and ligated into pJET1.2/blunt Cloning vector (Thermo Fisher Scientific, Erembodegem, Belgium) and transformed into *Escherichia coli* (DH5α) competent cells and then plated onto agar plates containing ampicillin (100 mg/ml) to generate a subtracted cDNA library.

Reverse northern dot-blotting for differential screening

Two hundred and ten cDNA clones were randomly selected from SSH library. The clones, freshly grown overnight at 37 °C were used as templates. The cDNA inserts were amplified by MyCycler™ thermocycler (Bio-Rad. Laboratories, Foster City, CA, USA) using nested PCR primers 1 and 2R, which were complementary to sequences flanking both ends of the cDNA insert. The 100 µl amplification reaction mixtures contained 78.7 µl sterile water, 10 µl of 10× reaction buffer, 0.75 µl of each primer (50 mM each), 2 µl dNTPs (10 mM each), 6 µl $MgCl_2$ (25 mM), 4 units of *Taq* DNA polymerase (Thermo Fisher Scientific, Erembodegem, Belgium), and 1 µl of bacterial culture. Thermocycling conditions were as follows: an initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.5 min, then a final extension at 72 °C for 5 min and held at 4 °C.

The PCR products were analyzed by 1.5 % agarose-gel electrophoresis. Amplified PCR products were spotted onto dry Hybond N^+ membranes (12 by 8 cm; Amersham Pharmacia Biotech, Little Chalfont, UK). DNA was bound to the nylon by soaking the membrane in 0.6 M NaOH for 5 min. Membranes were subsequently neutralized with 1.5 M NaCl in 0.5 M Tris–HCl pH 7.5 for 5 min and finally washed with a 2× SSC solution. Samples were cross-linked to membranes by baking for 2 h at 80 °C and then were stored at 4 °C for later use. First strand ^{32}P-dCTP cDNA probes were obtained by reverse transcription from 10 µg total RNA (derived from tester and driver samples) with Megaprime DNA labeling system (GE Healthcare, Buckinghamshire, UK) using random priming according to the furnisher's instructions. Membranes were pre-hybridized overnight at 65 °C in a solution containing 5× SSC, 5× Denhard's solution, 0.2 mg/ml sheared denatured salmon testes DNA, 0.005 M phosphate buffer pH 7 and 0.2 % SDS. Overnight hybridizations were performed at 65 °C in 2× SSC, 5× Denhard's, 0.005 M phosphate buffer, 0.2 % w/v SDS, 1 %, 0.2 µg/mL tRNA. Blots were washed three times for 25 min at 65 °C with 2, 1 and 0.1× SSC with 0.1 % (w/v) SDS at 65 °C, and then autoradiographed using a phosphor-imaging screens (Storage Phosphor Screen GP, Eastman Kodak, Rochester, NY, USA) and scanned using Personal FX (Bio-Rad, Hercules, CA, USA). Image analysis was undertaken using Quantity One software (Bio-Rad, Hercules, CA, USA).

DNA sequencing and data analysis

A total of 28 identified clones were sequenced by the GENOMELAB™ CEQ 8000/GenomeLab GeXPcapillary DNA analysis system (Beckman Coulter, Fullerton, CA, USA). To determine the function of these ESTs, nucleic acid and protein homology searches were performed using the BLASTx and BLASTn programs against the NCBI database (http://www.ncbi.nlm.nih.gov). *E*-values less than

Table 2 Primers used for the amplification of transcripts by semi-quantitative and quantitative PCR

Gene description (designation)	Forward primer sequence	Reverse primer sequence	Tm (°C)
ATP synthase CF1 alpha subunit (ATP synthase, JZ714621)	AAAGCATCTTCTGTGGCTCAA	GGCGCGAGGTATTGTAATGT	60
Chlorophyll a/b-binding protein (ChlBP, JZ714622)	CCTTTGGAGAACCTTGCTGA	TCCGGGAACAAAGTTGGTAG	57
Lipid transfer protein (LTP, JZ714623)	CAATGCTGTGGAGGAGTCAA	GCTGATTTCAAGCAGTTACAGG	56
Heat shock protein (HSP, JZ714624)	TTTTCAATATGGGTGCTGGTT	CTGCCAAGGCTTTTATCTGG	60
Zinc finger protein (ZFP, JZ714625)	CCACCGCTACTCCTTTCACT	GCATGAAGATTGATCCATACGA	58
Thioredoxin h (Trx h, JZ714626)	GAACAGCTCGAGAAGGGAAA	GCCAAAATTGGAGCAATGAA	57
Late embryogenesis abundant protein (LEA, JZ714627)	ACGAGTGGAGGAGTGGTGTC	CAGGATCAGGAACCCATGAA	60
Dicarboxylate transporter (Dct, JZ714628)	AAGTTGATTGGGTGGGTGGG	CGTAGTGATTTGCGAGCGG	58
Elongation factor 1 alpha (EF1α, AJ222579)	GACAACATGATTGAGAGGTCCACC	GGCTCCTTCTCAATCTCCTTACC	58

$1e^{-4}$ with more than 100 nucleotides in the ESTs were considered significant.

Semi-quantitative reverse transcriptase-PCR (RT-PCR)

Organ-specific expression of faba bean (Hara cultivar) selected genes was analyzed for leaves (4-week-old plants), fully opened flowers (0 days after podding), whole stems (2-week-old plants), whole roots (2-week-old plants), and cotyledons without testa (4-day-old seedlings). The total RNA was isolated using the protocol described by Chang et al. (1993). Five micrograms of RNA samples were treated with five units of RNase-free DNase I (Thermo Fisher Scientific, Erembodegem, Belgium) for 30 min at 37 °C to remove DNA contamination. The amount of total RNA was determined using NanoPhotometer® P-Class (Implen GmbH, Munich, Germany). The cDNA from each sample were synthesized using RevertAid M-MuLV Reverse Transcriptase (Thermo Fisher Scientific, Erembodegem, Belgium) following the manufacturer's protocol. The gene specific primer pairs (Table 2) were designed from assembled unigenes using Primer3 Input (version 0.4.0) software (Rozen and Skaletsky 2000) (http://frodo.wi.mit.edu/primer3/). The *EFα* gene from faba bean was used as an internal control to normalize differences between the loading amounts of the template. All PCR reactions were carried out within a final volume of 20 µl containing 1 µl of cDNA template, 0.5 µl of 10 mM dNTPs, 1.5 µl $MgCl_2$ (25 mM), 2 µl of 10× PCR buffer, 0.6 µl of each primer (10 µM), 0.2 µl of *Taq* polymerase (5 U/µl). The PCR parameters were set as follows: 30 cycles of 94 °C for 30 s, appropriate annealing temperatures for 30 s (Table 2), and 72 °C for 1 min, with an additional initial 5 min denaturation at 94 °C and a 5 min final extension at 72 °C.

Quantitative real-time polymerase chain reaction (q-RTPCR)

Real time RT-PCR was employed to validate the relative change in the expression of genes identified by SSH analysis. In the first time, the RNA samples from tester and driver initially isolated for the SSH analysis, and in the second time from Hara and Giza3 cultivars were used for real time RT-PCR.

After sowing, five seedlings from each cultivar were retained in five plastic pots filled with 5 kg of air-dried soil. For drought treatment, 15-day-old seedlings of Hara and Giza three cultivars were subjected to progressive drought by withholding irrigation. Experiments were conducted in a greenhouse receiving natural solar radiation, with air temperature regulated between 18 and 25 °C (night/day). Leaf tissues were harvested after 15-day-old seedlings (0 days water stress treatment), 19-day-old seedlings (4 days water stress treatment), 22-day-old seedlings (7 days water stress treatment), and 25-day-old seedlings (10 days water stress treatment). Total RNA was isolated using the protocol described by Chang et al. (1993). RNA was used as a template to synthesize the first strand cDNA with RevertAid M-MuLV Reverse Transcriptase (Thermo Fisher Scientific, Erembodegem, Belgium). The q-RT-PCR reactions were performed on an CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the SYBR Green dye method. The qRT-PCR reactions were performed in 25 µl volumes that included 12.5 µl of Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific, Erembodegem, Belgium), 100 ng of cDNA template and 1.0 µl each of the forward and reverse primers (10 µM). The PCR was done under the following conditions: 94 °C for 30 s, then 40 cycles of amplification at 94 °C for 30 s, 60 °C for 30 s. Each gene was normalized to the internal *EFα* levels. Each sample was run in triplicate to ensure quantitative accuracy, and the threshold cycle numbers (Ct) were averaged. The relative difference in expression was measured using the Basic ΔC_T method (Livak and Schmittgen 2001).

Results

Construction of suppression-subtracted cDNA library and analysis of subtraction efficiency

In this study, SSH was done to identify differentially expressed genes among cDNAs of leaves from unstressed (driver) and water stressed (tester) Hara cultivar. After extracting, the total RNAs were separated by agarose gel electrophoresis and two bright bands corresponding to 28S and 18S rRNA were visible (Fig. 1a).

The ratio A_{260}/A_{280} of the RNA from tester and driver ranged from 1.9 to 2 which showed that extracted RNA was pure. For double-stranded cDNAs synthesis procedure, 2 µg of mRNAs from tester and driver seedlings were reverse-transcribed and the cDNA was digested with *Rsa*I (Fig. 1b). cDNA before digestion with *Rsa*I, appeared as a smear of 0.5–4 kbp on 2 % agarose gel electrophoresis, and after digestion the cDNA size was smaller (0.1–2 kbp). After SSH, a primary and a secondary PCR were conducted to amplify those cDNAs that represented differentially expressed genes. Figure 1c showed obvious difference between the products of the first and the second-round PCR. The amplified bands mainly ranged from 200 to 1,500 bp, and from 200 to 1,000 bp for the primary and secondary PCR respectively.

We evaluated the subtraction efficiency by amplifying a housekeeping gene, *VfEFα*. The products obtained with 18,

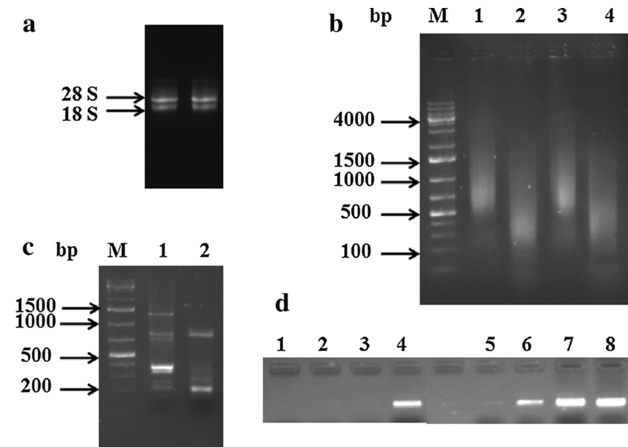

Fig. 1 Construction of a SSH library. **a** Electrophoresis of total RNA from driver (*lane 1*) and tester (*lane 2*) in 1 % agarose gel **b** analysis of tester and driver double-stranded cDNA before (*lane 1* and *3*) and after (*lane 2* and *4*) *Rsa*I digestion respectively. *Lane M* DNA size markers. **c** Comparison of subtractive products after the first-round PCR (*lane 1*) and the second-round PCR (*lane 2*). *Lane M* DNA size markers. **d** Evaluation of the subtraction efficiency by PCR using the housekeeping gene VfEFα: 1–4 were amplified from cDNA template before subtraction and 5–8 after subtraction. *Lanes 1* and *5* were the result of 18 amplification cycles, *lanes 2* and *6*, 23; *lanes 3* and *7*, 28; *lanes 4* and *8*, 33

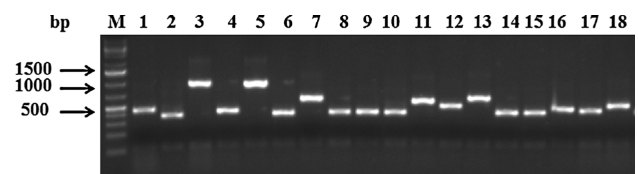

Fig. 2 PCR amplification of positive clones randomly picked up from the SSH library. There was a average insert size of 300–1,000 bp. *Lanes 1–18* PCR products from different clones. *Lane M* DNA size markers

23, 28 and 33 cycles were separated on 1 % agarose gel electrophoresis (Fig. 1d).

For the samples before subtraction, the housekeeping gene *VfEFα* could be detected after 23 cycles of amplification. However, for the samples after subtraction, *VfEFα* could only be detected after 33 cycles of amplification (ten cycles later; Fig. 1d). These data suggest that the subtractive library was of high quality.

PCR amplification and differential screening by dot-blot hybridization

The second PCR products of SSH were cloned into pGEM-T vector after purification, and transformed into *E. coli*. A total of 480 positive clones were found in the blue/white selection, and 210 out of them were randomly selected for colony PCR (Fig. 2). 91 % of them were effectively recombinant and the inserts with estimated size between 300 and 1,000 bp were selected for dot blot hybridization. A set of two identical nylon membranes was prepared for dot-blot hybridization to be hybridized with Tester and driver cDNA ^{32}P-labeled probes. Clones detected with different signal intensity with the tester probe compared with driver probe were considered to be water stress related genes. A total of 24 clones hybridized with the tester probe were found to be up-regulated, and considered to be water stress induced tolerance-related genes and four clones appeared to be water stress down-regulated genes (Fig. 3).

Sequence and homology analyses of SSH cDNA sequences

The 32 water stress related clones were sequenced in order to identify putative key genes related to regulation and water stress tolerance in this species. The EST cluster analysis indicated that these sequences represented ten unique ESTs. All the unique ESTs were submitted to the EST database of Gen-Bank http://www.ncbi.nlm.nih.gov/dbEST. Based on BLASTx and BLASTn homology search, among the ten non-redundant sequences, nine sequences are homologous to known genes and the remaining one sequence is homologous to genes with unknown function (Table 3). Based on gene ontology (GO) annotation, the nine unique ESTs with significant homology (*E*-value $< 1e^{-04}$) were divided into four organizing principal GO categories: photosynthesis, abiotic stress response, expression and regulation, and energy metabolism.

Annotation results showed that this library contained several genes previously reported to be involved in cellular stress, such as heat shock protein (*HSP*), late embryogenesis abundant protein (*LEA*), and chlorophyll a/b-binding protein (*ChlBP*). More noteworthy was the identification of the transcription factor *ZFP* (zinc finger protein), which is related to several plant-specific biological processes, such as water stress tolerance. The library also included cDNAs not previously reported to be associated with water stress response, such as those coding for the dicarboxylate transporter protein (*Dct*).

The large proportion of the annotated genes represented by single or two ESTs (6 of 10, i.e. 60 %) seems to indicate an efficient normalisation of the libraries; neither of the genes was represented by more than five ESTs. Furthermore, only two ESTs (Vf_SSh9 and Vf_SSh10) of the annotated genes in the different functional groups were represented by ESTs detected from both tester and driver probes.

Organ-dependent expression of the selected ESTs

As an initial characterization of gene expression in faba bean plants, an expression pattern of eight selected ESTs

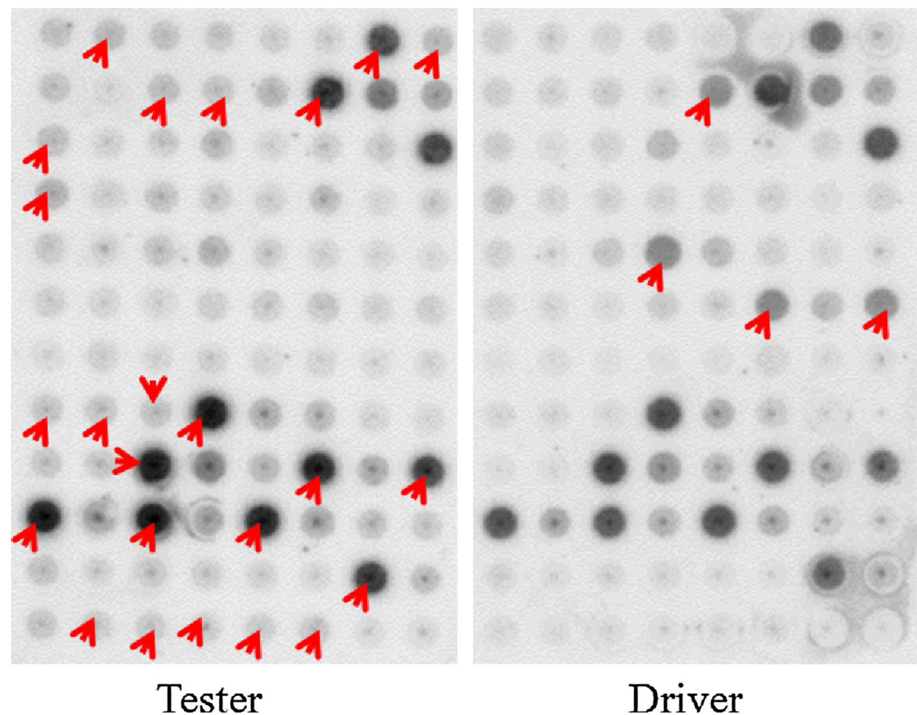

Fig. 3 Differential screening of clones from subtracted library by dot-blot analysis using tester and driver cDNA as probes. Differentially expressed clones are marked with *arrows*

Table 3 Putative identities and characteristics of the sequences selected by SSH

Clone	Length (bp)	Number of identical clones	Homology analysis	Putative identity (accession no.)	Identities (aa/aa or bp/bp)	*E*-value	Accession no. of EST in Genbank
Vf_SSh1	264	3	BLASTn	ATP synthase CF1 alpha subunit (*Vigna unguiculata*) AM748481	245/265 (92 %)	2E−101	JZ714621
Vf_SSh2	274	2	BLASTn	Chlorophyll a/b-binding protein (*Pisum sativum*) J01253	248/274 (91 %)	2E−101	JZ714622
Vf_SSh3	225	2	BLASTn	Lipid-transfer protein (*Medicago truncatula*) XM_003605221	172/223 (77 %)	1E−39	JZ714623
Vf_SSh4	155	2	BLASTn	Heat shock 70 kDa protein (*Cicer arietinum*) XM_004511300	145/155 (94 %)	7E−59	JZ714624
Vf_SSh5	187	1	BLASTn	Zinc finger protein (*Medicago sativa*) Y18788	167/185 (90 %)	3E−64	JZ714625
Vf_SSh6	191	2	BLASTn	Thioredoxin h (*Pisum sativum*) AY170650	178/191 (93 %)	5E−74	JZ714626
Vf_SSh7	186	2	BLASTx	Late embryogenesis abundant protein LEA-3 (*Ammopiptanthus mongolicus*) AAW31666.1	50/78 (64 %)	1E−21	JZ714627
Vf_SSh8	117	4	BLASTx	Putative dicarboxylate transporter (*Algoriphagus* sp) WP_008202460	14/37 (38 %)	0.68	JZ714628
Vf_SSh9	190	5	BLASTx	Uncharacterized protein (*Solanum lycopersicum*) XP_004246818	33/65 (51 %)	8E−18	JZ714629
Vf_SSh10	359	5	BLASTx	Gag/pol polyprotein (*Pisum sativum*) AAQ82037.1	29/45 (64 %)	9E−13	JZ714630

[lipid transfer protein (*LTP*), dicarboxylate transporter (*Dct*), *ATP synthase*, thioredoxin h (*Trx h*), *ZFP*, *HSP*, *ChlBP*, and *LEA*] listed in Table 3 was examined using RT-PCR in different organs (Fig. 4). Total RNA was isolated from roots, flowers, stems, cotyledons, and leaves from faba bean plants. The majority of the studied genes were found expressed at various levels in the different organs tested. In general, the expression level of all

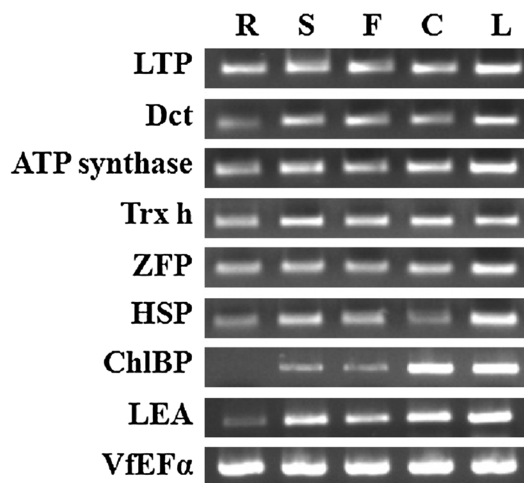

Fig. 4 RT-PCR of selected genes from faba bean tissues: LTP (JZ714623), DCt (JZ714628), ATP synthase (JZ714621),Trx h (JZ714626), ZFP (JZ714625), HSP (JZ714624),ChlBP (JZ714622), and LEA (JZ714627). Roots (R), Stems (S), Flowers (F), Cotyledons (C) and Leaves (L). *Lower lanes* faba bean EFα internal control

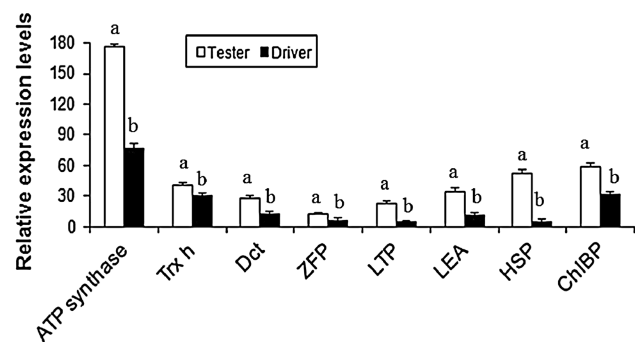

Fig. 5 Relative expression ratios of the transcripts of eight selected SSH candidates in leaf tissues in the driver and tester. ATP synthase (JZ714621), Trx h (JZ714626), Dct (JZ714628), ZFP (JZ714625), LTP (JZ714623), LEA (JZ714627), HSP (JZ714624), and ChlBP (JZ714622). The relative mRNA levels of individual selected genes were normalized to the housekeeping gene EFα. Data shown represent mean values obtained from three independent amplification reactions and the *error bars* indicate SD. Significant difference ($P < 0.05$) detected by Tukey's multiple comparison test is shown by *different letters* above the *bars*

selected genes was significantly higher in leaf tissues than in any other tissues. The expression level of *LEA* was relatively high in cotyledons and leaves compared to the expression levels in other tissues. As expected, the expression of *ChlBP* was barely detected in the root tissues and a low level expression was observed in stem and flower tissues, while the expression levels of *ChlBP* in cotyledon and leaf tissues were very high. RT-PCR showed that both *HSP* and *Dct* mRNA levels were slightly present in root and cotyledon tissues, well detected in steam and flower tissues; however, in leaf tissues the abundance of the *HSP* and *Dct* mRNA appeared to be higher.

Confirmation of the differential expression of selected genes by real time PCR

In order to confirm that genes identified by SSH are differentially expressed in tester compared to driver, real-time PCR was conducted in parallel to verify the validity of the SSH data and dot-blot analysis. Eight genes (*ATP synthase*, *Trx h*, *HSP*, *ZFP*, *LTP*, *LEA*, *Dct* and *ChlBP*) of faba bean identified from SSH library were selected, and the expression of these genes was determined by real-time PCR using specific sets of primers (Fig. 5). The 8 genes selected for validation were chosen because they had previously been associated with cellular response to biotic and abiotic stress, or because of their possible contribution to tolerance to water stress. The transcription levels of mRNA were significantly higher for all the tested eight genes in tester, compared with driver tissues (controls). Real-time PCR data (Fig. 5) revealed that ESTs with significant homologies to *HSP*, *LTP*, *LEA*, *ATP synthase*, *Dct*, *ChlBP*, *ZFP* and the putative protein encoding to *Trx h* were 9.13, 4.62, 3.05, 2.29, 2.18, 1.84, 1,79 and 1.32 times over-expressed, respectively, in the tester compared with driver.

Expression analysis of selected ESTs during water stress by real-time PCR

Regulatory genes are expected to perform crucial functions in tolerance to water stress. With a particular interest in these genes, six genes (*Trx h*, *LEA*, *HSP*, *LTP*, *ZFP*, and *ChlBP*) were preferentially chosen based on their putative annotations linked to transcription, protein metabolism, defense/stress response and signaling to verify the differences in the gene expression between the Hara and Giza 3 cultivar. Abdellatif et al. (2012) suggested that Giza 3 could be considered as susceptible faba bean variety for drought stress. The expression patterns of these genes were quantified by quantitative RT-PCR, using total RNA isolated from leaves at 0, 4, 7 and 10 days water stress (Fig. 6). All of these genes showed a changed level of expression in response to water stress. Indeed, expression of the mRNA of *Trx h*, *LEA* and *ChlBP* in Hara was significantly increased after the water stress particularly at T1, whereas *HSP* at T2 compared to T0. Expression of *LTP* was significantly increased at T1 and T2. The transcript level of the gene encoding a putative *ZFP* transcription factor was significantly decreased at T1 and T2, but reached a peak at T3 compared to T0. In general, the results indicated that the expression of the selected genes in Giza 3 was significantly less, or showed similar patterns of expression as in Hara.

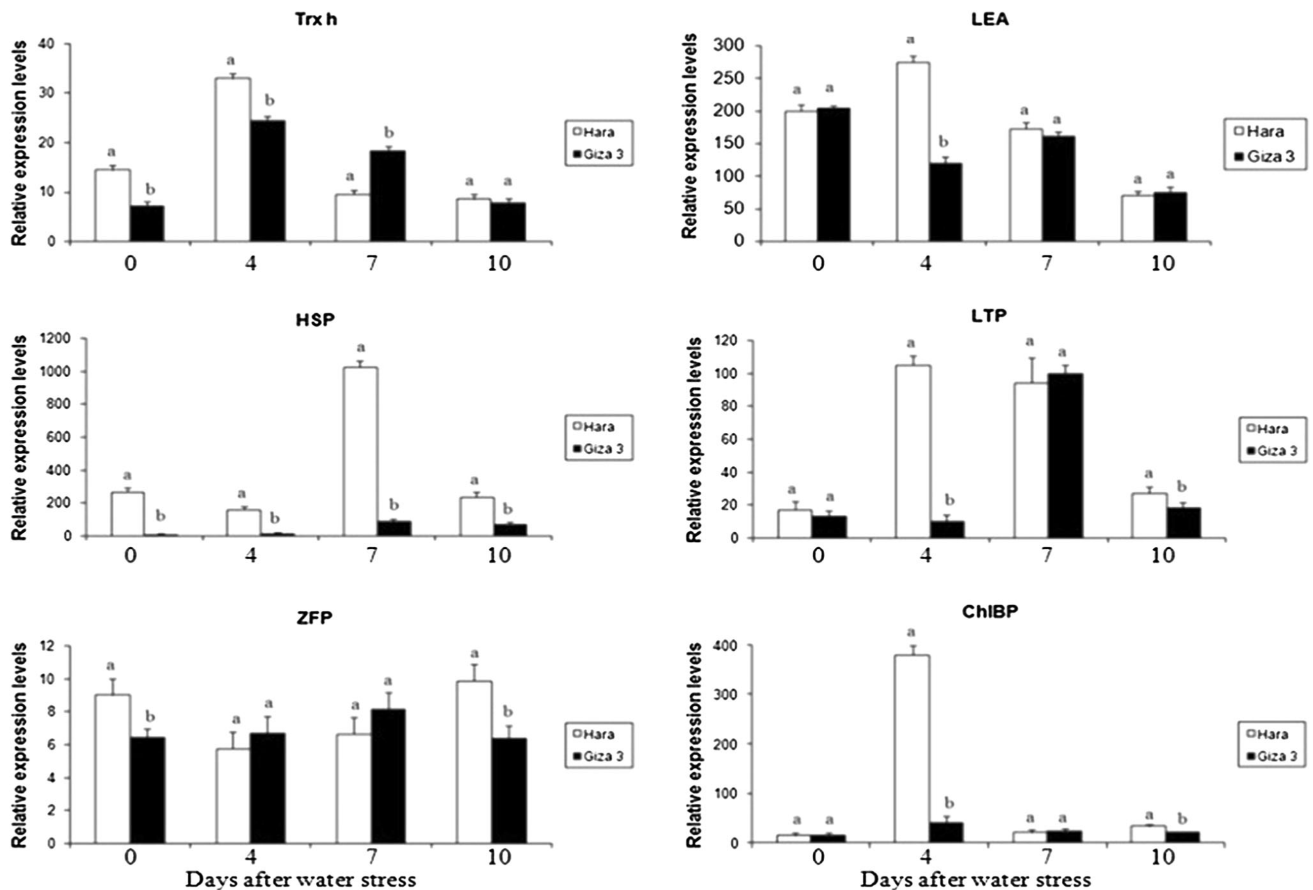

Fig. 6 Relative expression levels of six genes (Trx h, LEA, HSP, LTP, ZFP, ChlBP) in four developmental stages during water stress treatments by real time PCR in Hara and Giza 3 cultivars. The four stages are: T0 (not stressed plants), T1 (4 days water stressed plants), T2 (7 days water stressed plants), and T3 (10 days water stressed plants). Data were normalized with respect to the housekeeping gene VfEFα. Significant differences ($P < 0.05$) detected by Tukey's multiple comparison test are shown by *different letters* above the *bars*

Discussion

Faba bean is a major food legume crop grown in Tunisia. It is used for human consumption in a wide range of traditional dishes, as well as for animal feed (Kharrat and Ouchari 2011). Its production, however, is low (1.38 t/ha for the small seed and 1.03 t/ha for the large seed) due to various factors of which drought is becoming very important due to its frequency in the recent past. Considering the importance of the faba bean crop and water deficit as an environmental factor limiting its production, it is necessary to identify and characterize candidate genes involved in the response to water deficit to obtain more tolerant varieties. Drought is among the major abiotic environmental stress that limit plant growth and development (Sapeta et al. 2013). Plants respond and adapt to environmental stresses such as drought at physiological and biochemical levels by the induction of both regulatory and functional sets of genes (Cheng et al. 2013a).

To identify key water stress-related genes in faba bean, in order to understand the molecular mechanisms of tolerance to water stress, a cDNA library containing water-stress induced transcripts in faba bean leaves was constructed through SSH and the function of differentially expressed genes induced by water stress was analysed.

Ten differentially expressed transcripts were examined. Eight transcripts showed homology with previously described genes from plant species such as *Medicago truncatula*, *Medicago sativa*, *Pisum sativum*, *Vigna unguiculata*, and *Cicer arietinum*. One transcript showed great homology with uncharacterized proteins in *Solanum lycopersicum*. Based on our bioinformatics analysis, this SSH cDNA library contained several genes related to drought stress tolerance previously reported in Arabidopsis and maize (Seki et al. 2002; Luo et al. 2010).

Expression of eight genes in various faba bean tissues was analysed by semi-quantitative RT-PCR. The results showed that expression levels of these genes were generally high in leaves. Some genes were expressed at a high

level in non-leaves tissues such as cotyledon. These newly identified genes showed different spatial expression patterns and each member might play particular physiological functions.

In the current study, a TFIIIA-type Zn finger protein (JZ714625) was identified. The TFIIIA-type zinc finger protein genes (*ZFPs*), one of the largest families of transcriptional regulators have been revealed to be required for key cellular processes such as responses to drought stress (Zhang et al. 2012a). *ZFPs* enhance the activities of reactive oxygen species-scavenging enzymes under stress conditions and increased tolerance of plants to oxidative stress. In rice leaves *ZFP182* is involved in ABA-induced up-regulation in the activities of SOD (superoxide dismutase) and APX (ascorbate peroxidase) (Zhang et al. 2012b). Xu et al. (2008) and Liu et al. (2013a) found that overexpression of *ZFP252* and *DgZFP3* in rice and tobacco increased tolerance to drought stress. In our study, the transcript of this gene showed the highest expression level at T3 (10 days water stressed plants) and revealed higher expression in Hara than Giza 3 (sensitive cultivar). The relatively increased expression of *ZFP* indicated that the gene may be required for the drought stress tolerance in faba bean. This should be the first report that *ZFP* gene is differentially induced during drought stress in different faba bean cultivars.

Heat-shock proteins (*HSPs*) are found among the genes that have been successful in improving drought tolerance. *HSPs* such as *HSP70* and *HSP90* play key roles in drought stress signal transduction and consequently protecting plants from water stress by re-establishing normal protein conformation and thus cellular homeostasis (Wang et al. 2004). Over-expression of *HSP70* in tobacco was correlated with maintenance of optimum water content suggesting that elevated level of *NtHSP70-1* is related to an adaptive stress response conferring drought tolerance in tobacco plants (Cho and Hong 2006). The expression of *HSP* gene in faba bean was shown to be up-regulated during drought stress in Hara cultivar, particularly at T2 (7 days water stressed plants), while its expression level is very lower in Giza 3 at all tested stages. Faba bean mitochondrial *HSP70* seems therefore to play a role during high levels of stress. During severe stresses, when the levels of ROS (reactive oxygen species) are higher, the increased expression of mitochondrial *HSP70* might be due to an increase in refolding/transport of antioxidant proteins to the mitochondria (Cruz de Carvalho 2008). Interestingly, it has been shown that mitochondrial *HSPs* protects the NADH:ubiquinone oxidoreductase complex during heat stress in plants (Downs and Heckathorn 1998). Indeed, the increased expression of this gene might be due to an increased need of protection of this complex due to the overproduction of ROS in the mitochondrion (Cruz de Carvalho 2008).

Our results showed that the *HSP* gene was highly expressed at T2 (7 days after water stress), therefore we supposed that it might play important functions in faba bean leaves tissues. Indeed, faba bean *HSP* may help to protect the mitochondrion reduce, repair, or protect against oxidation damage due to the severe drought stress applied.

Plants induce expression of a number of specific genes in response to drought stress such as late embryogenesis abundant (*LEA*) proteins. These genes may play a role in stabilization of membrane structures and protected macromolecules (Battaglia and Covarrubias 2013).

The over-expression of *LEA* genes in rice and poplar showed enhanced drought stress resistance in these species by mediating some physiological processes associated with drought tolerance of plants (Xiao et al. 2007; Gao et al. 2013). In our experiments, mRNAs corresponding to *LEA* were present in all tested samples and their amount was related to the degree of water deficit (higher in plants treated at T1 but decreased at T2 and T3). We suggest that the expression of *LEA* by water stress treatment was time-dependant. Moreover, *LEA* was more expressed in the Hara cultivar than in the susceptible cultivar (Giza 3). All together, the results indicate that in faba bean the *LEA* gene could be regulated in response to abiotic stresses.

Lipid transfer proteins (*LTPs*) are ubiquitous in plants and are encoded by multigene families that are involved in developmental and stress response processes (Wang et al. 2009). Federico et al. (2005) found that abscisic acid induced *LTP* genes expression. These results indicated that *LTP* could play an important role in abiotic stress tolerance.

The relative abundance of *LTP* in other organ was much lower than in leaves, indicating that the physiological importance of the gene in leaves is higher than in other organs. In leaves, qRT-PCR showed that the *LTP* gene was differentially regulated under water stress suggesting a role in water stress response. Interestingly, it was highly up-regulated (tenfold) in the leaf tissue of Hara compared to Giza 3 at T1, suggesting that faba bean *LTP* elicit drought tolerance early during a period of stress. Previous studies have shown the up-regulation of some *LTP* genes by drought stress (Gonorazky et al. 2005). Furthermore, over-expression of *LTP* gene conferred drought tolerance, in Arabidopsis (Jung et al. 2005).

Photosynthesis and cell growth are the first processes to be affected by water deficit (Chaves et al. 2009). Light-harvesting chlorophyll a/b-binding protein (*LHCB*) is one of the major chloroplast proteins in plants required for photosynthesis (Xia et al. 2012). Interestingly, Xu et al. (2012) found that *LHCBs* are member of Chlorophyll a/b-binding protein family involved in ABA signaling and suggested that they are required for stomatal response and modulating ROS homeostasis in Arabidopsis.

In faba bean this gene was differentially expressed at different water stress levels. Although this gene was expressed in

Giza 3 (sensitive cultivar), it was increased 9.57 times in Hara at T1, which means that both plants are using this gene in their process of drought stress response. The difference lies in the fact that in the Hara plant this gene may be required particularly at T1, whereas the Giza 3 plant follow the same pattern of Hara as the stress becomes more severe.

Thioredoxins h play a role in many important biological processes such as germination and early seedling growth (Cazalis et al. 2006). Furthermore, thioredoxins h could be involved in the cellular protection against oxidative stress, in particular at the beginning of the desiccation phase during seed development (Serrato and Cejudo 2003). In soybean, expression of thioredoxin h (*GmTrx*) showed reduced reactive oxygen species levels during nodule development (Lee et al. 2005). In bacteria, yeast and mammals, thioredoxins play an essential role in the response to oxidative stress. These data are in agreement with our results in faba bean. Without any stress (T0) the tolerant cultivar, Hara, showed a higher expression of *Trx h* transcripts compared to Giza 3, suggesting that *Trx h* expression was cultivar dependant. The water stress treatment promoted an increase of *Trx h* transcripts at T1 in Hara compared to Giza 3, but expression was not significantly affected at T3.

Thus, we suggest that in faba bean water deficit results in substantial changes in the chloroplastic redox state leading to oxidative damage. Induction of *Trx h* by water stress may result from changes in the chloroplast redox state and we propose that the protein participates in the response to oxidative stress within chloroplast upon water deficit in faba bean. Indeed, *Trx h* may either regenerate proteins inactivated by redox change or supply electrons to a thioredoxin-dependent protein involved in scavenging of peroxides.

Similar results were obtained by Broin et al. (2000) which showed that accumulations of *CDSP 32*, a drought-induced thioredoxin, were revealed upon oxidative treatments in potato plants. These authors found that *CDSP 32* may preserve chloroplastic structures against oxidative injury upon drought.

All the drought-related genes considered in this experiment showed a differentially and higher expression in Hara than in Giza 3 particularly at T1 proving that the two cultivars have a different ability to induce the drought molecular response. The *Trx h*, *LEA*, *LTP* and *ChlBP* genes have significantly increased in expression after the plants were subjected to 4 days of stress (T1) in Hara cultivar compared to Giza 3, although this increase was significant in HSP and ZFP genes at T2 and T3 respectively.

Conclusion

An SSH library for drought tolerance constructed in faba bean allowed cloning genes that are specifically up- and down-regulated in the leaves in response to water stress. Annotation of the ESTs predicted that most of them encoded proteins involved in transcriptional regulation, stress response, biogenesis and photosynthesis. Moreover, we identified eight novel genes that are associated with drought stress response. This study also provides a comparative overview of genotype-specific expression patterns of these genes in different organ tissues of faba bean and in response to water stress. The up-regulation of some identified genes was confirmed by real-time qPCR. Results from this study could serve as resource for marker discovery and can provide information for the appropriate selection of candidate genes associated with drought tolerance and may help in targeting useful genes for improving drought tolerance in faba bean. To convert these putative genes into candidate genes for genetic improvement of faba bean, further characterization is needed.

The development of new genetic materials such as a segregant population for QTL analysis, drought resistant/susceptible mutants could serve as a foundation for future studies into the elucidation of the drought stress response mechanisms of faba bean. Moreover, some important identified genes (*LEA*, *HSP* and *ZFP*) might be candidate genes in further study and transgenic engineering. Thus, it would be interesting to get their corresponding full-length sequence, in order to analyze their precise function in drought stress tolerance in faba bean. Interestingly, several efficient plant regeneration protocol have been developed for biotechnological breeding of economically important legume crops such as soybean (Arun et al. 2014) and chickpea (Tripathi et al. 2013). Indeed, overexpression of these genes would contribute to a better understanding of the molecular mechanisms of signal transduction pathways, which could lead to improve faba bean drought stress tolerance and establish the necessary framework of knowledge for the advancement in genetic transformation and regeneration of this recalcitrant grain legume specie.

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