

Isolation of cowpea genes conferring drought tolerance: Construction of a cDNA drought expression library[#]

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Abstract

The main objective of this study was to identify and isolate the genes conferring drought tolerance in cowpea. A cDNA library enriched for cowpea genes expressed specifically during responses to drought was constructed. A procedure called suppression subtractive hybridisation (SSH) was successfully employed to obtain these differentially expressed transcripts. The library consists of 4 160 individual clones. Preliminary sequencing results identified two clones to be known stress-related plant genes (GST (glutathione-S-transferase) and PR-1 (pathogenesis-related protein-1)). Micro-array analysis will be performed on the library's clones to verify their differential expression during drought conditions, and to identify additional clones for further investigation. Promising genes can eventually be used to genetically engineer crops for drought tolerance to improve food security in water-stressed areas.

Keywords: cowpea, drought tolerance, suppression subtractive hybridisation (SSH)

Introduction

South Africa is classified as a water-scarce country (Department of Water Affairs and Forestry (DWAf), 2004). The utilisation of marginal soils by plants tolerant to drought stress provides the greatest potential for agricultural expansion. Drought stress is, however, a multigene trait. Therefore, breeding for tolerance must include different strategies, including gene transfer. However, in order to understand the gene networks that underlie plant stress, it is necessary to identify and characterise the genes that respond to abiotic and biotic stress.

Cowpea (*Vigna unguiculata* L. Walp) is indigenous to Southern Africa. Most lines are drought-tolerant and can be grown under the harshest growing conditions. It is, therefore, an important crop for subsistence and small-holder farmers (Quass, 1995).

The aim of this research project was to identify and isolate the genes conferring drought tolerance in cowpea. These identified drought-tolerant genes will be used in the transformation of crops of importance for the resource-poor farmer, leading to increased food security.

The first step in this quest, as well as in many other biological investigations, is to construct a cDNA library enriched for differentially expressed transcripts. Successful projects that followed a similar approach include the characterisation of the defence pathways in pearl millet and *Fusarium* wilt in banana (Van den Berg et al., 2004). This paper addresses the construction of a customised cDNA library that contains cowpea genes expressed specifically during responses to drought. A procedure called suppression subtractive hybridisation (SSH) was

employed to obtain these differentially expressed transcripts (Diatchenko et al., 1996). The completed library can also serve as a valuable resource in the investigations of other characteristics of cowpea.

Materials and methods

Cowpea drought trial

A cowpea drought trial was performed in which one drought-tolerant (IT96D-602) and one susceptible (Tvu7778) cowpea line were subjected to drought conditions. Physiological parameters of plant stress were measured in order to determine time points for the preparation of the drought expression library.

Suppression subtractive hybridisation (SSH)

Differential expression analysis by means of SSH (Diatchenko et al., 1996) was employed to prepare a cDNA drought expression library for cowpea. Unstressed susceptible cowpea (Tvu7778) cDNA was subtracted from drought-stressed tolerant cowpea (IT96D-602) cDNA as previously described (Van den Berg et al., 2004; Hein et al., 2004). Briefly, RNA was isolated from unstressed Tvu7778 (9 and 12 d) and drought-stressed IT96D-602 (9 and 12 d without water) using Tri-reagent (Sigma) and PVP (Ambion's Plant RNA isolation aid). Contaminating genomic DNA was removed with the Turbo DNA-free kit (Ambion) and the RNA cleaned up with the Plant RNeasy kit (Qiagen). mRNA was isolated from 50 µg pools of stressed IT96D-602 RNA (tester) and control Tvu7778 RNA (driver) using an Oligotex mRNA purification kit (Qiagen). cDNA was synthesised from mRNA using the Roche cDNA synthesis kit.

Subtractive hybridisation was performed on *RsaI* digested cDNA fragments using the PCR-Select cDNA subtraction kit (Clontech). Both forward and reverse subtractions were performed. After subtraction the products were amplified by a primary PCR and a nested secondary suppression PCR to generate differentially expressed cDNA fragments.

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Preparation of a drought expression library

PCR products from six individual reactions were pooled, separated on an agarose gel and DNA fragments excised in three size ranges, namely <200 bp, 200 to 650 bp and 650 to 1 700 bp. The fragments were purified from the gel using the Qiaquick gel extraction kit (Qiagen).

Fifty nanograms of each cDNA fraction were ligated into the pGEM-T Easy Cloning Vector and transformed into *Escherichia coli* JM109 following the manufacturers' instructions (Promega). Transformation reactions were plated out on blue-white selection media (140 mm LB-agar plates containing 100 µg/ml ampicillin, spread with 20 µl X-Gal (50 mg/ml in DMF) and 50 µl IPTG (0.2 M)). The plates were incubated at 37°C for 16 h after which they were stored at 4°C for 24 h. White colonies were picked, grown overnight at 37°C in liquid LB medium containing 100 µg/ml ampicillin and stored as 25% glycerol stocks at -70°C in sterile 96-well microtitre plates (Corning).

Sequencing of selected clones

Randomly selected clones of the forward subtracted library were sequenced using the SP6 and T7 primers and BLAST (Basic Local Alignment Search Tool; Altschul et al., 1990) analysis performed to identify possible drought-tolerance genes.

Preparation of amplified drought expression library cDNA fragments

Library PCR amplification: A PCR master mix, containing 1× Ammonium acetate buffer, 2.5 mM MgCl₂, 200 µM dNTPs, 0.25 µM each of SP6 (5'- ATT TAG GTG ACA CTA TAG -3') and T7 (5'- TAA TAC GAC TCA CTA TAG GG -3') primers, and 1U Biotaq DNA polymerase (Bioline) in a total volume of 100 µl nuclease-free water, was prepared in a sterile plastic tube. One hundred µl aliquots were added to each well of a 96-well PCR plate (Thermo-Fast, ABGene). The PCR plates were inoculated with the overnight bacterial cultures of cDNA clones using a 96-pin plate replicator (Nunc). The PCR plate was sealed with a silicon mat (Corning).

PCR cycling conditions: Reactions were incubated in a PTC-100 Thermocycler (MJ Research) at 94°C for 5 min, followed by

30 cycles each of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min. A final elongation step of 72°C for 5 min was included. The PCR products were stored at 4°C until purification.

PCR clean-up: PCR products were cleaned up with Montage PCR purification plates (Microsep). PCR samples (100 µl) were transferred to the wells of the Montage PCR purification plate, placed on a vacuum manifold (Microsep) and a vacuum of 20 inch Hg applied for 20 min. The plate was blot-dried using paper towels. Fifty µl of MilliQ water were applied to each well and the plate agitated for 15 min at 1 400 r/min on an Ika-Vibrax VXR shaker (Janke & Kunkel Typ VX1). The suspensions were transferred to a 96-well storage plate and covered with well caps (Nunc). The purified PCR products were stored in the storage plates at -20°C.

Stretch gel: PCR products were analysed on a 1% agarose gel in 0.5× TAE containing ethidium bromide using the ElectroFast Stretch 204 gel assembly (ABGene). Samples were loaded with 1× ReddyRun gel loading buffer (ABGene) and 3 µl (75 ng) of ElectroFast marker (ABGene) were loaded into the first lane of each row. A voltage of 100V was applied for 30 min and the gel visualised and photographed on a UV source.

Results and discussion

Cowpea drought trial

The two cowpea cultivars IT96D-602 and Tvu7778 were chosen for this study, since previous studies had established their respective drought tolerance and susceptibility (Spreeth et al., 2004). Physiological data were collected during a drought trial, of which total leaf proline content is presented in Fig. 1.

It has previously been determined that proline content gives an indication of the level of stress experienced by a plant (Spreeth et al., 2004). Proline content increased at a slower rate and to a lower concentration in the drought tolerant line IT96D-602 than in the susceptible line Tvu7778 (Fig. 1). A lower proline content is a good indication that a plant is coping well during drought stress. IT96D-602 also showed better recovery at day 16 when re-watered.

Physiological variance accounts for the high variability

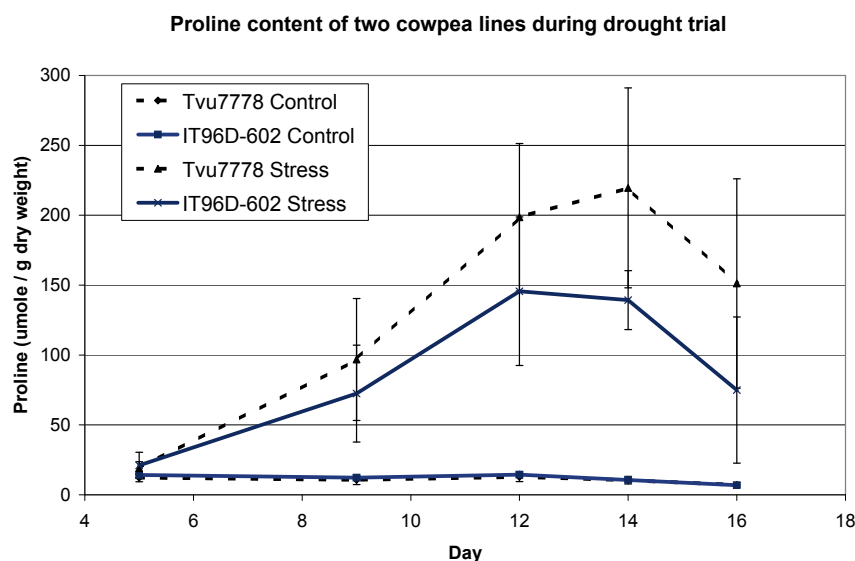


Figure 1
Proline content in µmole per gram dry leaf weight of a susceptible (Tvu7778) and a tolerant (IT96D-602) cowpea line during the 16-day drought trial planted in October 2004 at the Agricultural Research Council – Vegetable and Ornamental Plant Institute (Roodeplaat). The average and standard error of five biological replicates are presented.

observed (Fig. 1), which is characteristic of biological systems. Days 9 and 12 were chosen as the time points to isolate RNA for generation of the drought expression library.

Suppression subtractive hybridisation (SSH)

Forward and reverse subtraction of drought stressed IT96D-602 and unstressed Tvu7778 cDNA were performed. Genes that are expressed and up-regulated due to the drought tolerant phenotype, general stress responses, as well as genotypic differences between the two lines, were amplified during SSH. SSH also equalises the abundance of cDNAs in the target (Diatchenko et al., 1996), which increases the likelihood of low-copy genes (rare transcripts) being incorporated into the library.

Figure 2 presents the distinct patterns of PCR products obtained from six individual nested secondary PCR for each of the forward and reverse subtractions (Fig. 2, lanes 1-12). The secondary PCRs were performed using separate primary PCRs as templates. Discrete bands were observed in the subtracted samples (Fig. 2, lanes 1-12, pooled together in lane 16 and 17), while the un-subtracted tester (UT) and driver (UD) PCR yielded a smear (Fig. 2, lanes 14-15). The subtracted tester (ST, Fig. 2, lane 16) refers to the forward subtraction and the subtracted driver (SD, Fig. 2, lane 17) to the reverse subtraction.

The presence of a number of distinct bands in the individual subtracted samples (Fig. 2, lanes 1-12) is an indication that the SSH was successful, since they represent genes that have been enriched for. A smear represents genes that weren't enriched during the SSH procedure.

The forward and reverse subtracted cDNA fragments, ranging from 0.1-2kb in size, were cloned to produce the cDNA library.

Preparation of drought expression library

A cDNA library consisting of 44 96-well microtitre plates, containing 4 160 individual clones, was generated. This library is expected to contain the genes that are involved in drought tolerance, which is an important outcome of this project.

Sequencing of selected clones

BLAST analysis was performed on the sequences of five randomly selected cowpea clones. The alignments that gave the smallest e-values corresponded to genes deposited in the BLASTX database (Table 1 – see next page).

Four out of the five selected cowpea cDNA clones coded for known genes found in a variety of plant species. Two are known to be stress-related genes (GST (Glutathione-S-transferase) and PR-1 (pathogenesis related protein-1)). Additional clones will be selected and sequenced in future.

Since cowpea is not a model plant, sequence data available on databases are limited. Therefore, only cowpea genes that are conserved in other plants of which sequence information is available, such as *Arabidopsis thaliana* whose genome has been sequenced, can be given identities based on their homology. However, even *A. thaliana*'s genome is not as yet fully annotated, which, in this study, may result in the classification of an interesting clone (such as 4A1 in this case) as an unknown putative protein.

Preparation of amplified drought expression library cDNA fragments

The 4160 clones were amplified by PCR and the products purified as described in the Materials and Methods. The stretch gel

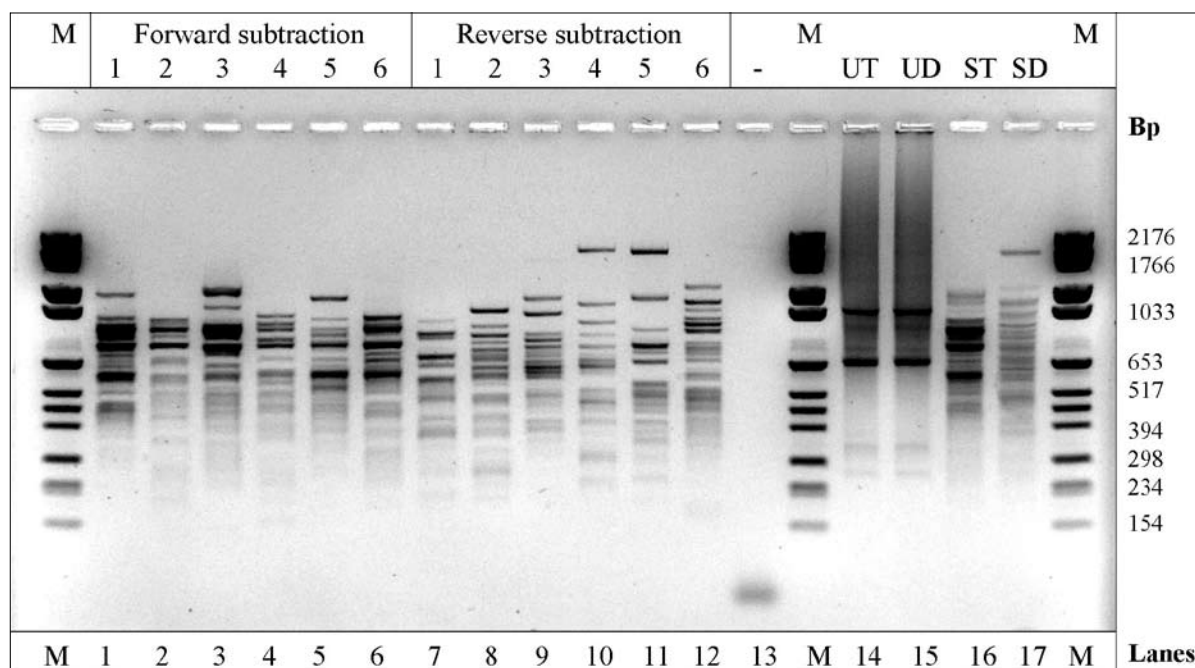


Figure 2

Secondary PCR amplification of subtracted and unsubtracted cDNA fragments produced from drought stressed IT96D-602 and unstressed Tvu7778 cowpea cultivars that had been subtracted with suppression subtractive hybridisation. M: Molecular weight marker VI (Roche; 154-2176bp). The secondary PCR products were loaded as follows: Lanes 1-6: six individual subtracted tester (ST) samples; Lanes 7-12: six individual subtracted driver (SD) samples; Lane 13: negative water control; Lanes 14-15: pooled unsubtracted tester (UT) and unsubtracted driver (UD), respectively; Lane 16-17: pooled subtracted tester (ST) and subtracted driver (SD), respectively.

Clone	Putative gene	Share similarity to genes in plant species	Best e-value	Stress-related
2A1	MtN19	<i>Pisum sativum</i> , <i>Medicago truncatula</i> , <i>Oryza sativa</i> , <i>Arabidopsis thaliana</i>	4E-73	?
2A2	GST	<i>Phaseolus acutifolius</i> , <i>Glycine max</i> , <i>Arabidopsis thaliana</i> , <i>Vitis vinifera</i> , <i>Citrus sinensis</i>	9E-95	Yes
3A2	miraculin, tumor-related protein	<i>Solanum melongena</i> , <i>Nicotiana tabacum</i>	3E-12	?
3A6	PR-1	<i>Glycine max</i> , <i>Vitis vinifera</i> , <i>Arabidopsis thaliana</i> , <i>Brassica napus</i> , <i>B. juncea</i> , <i>Nicotiana tabacum</i>	3E-58	Yes
4A1	unknown protein	<i>Arabidopsis thaliana</i>	5E-15	?

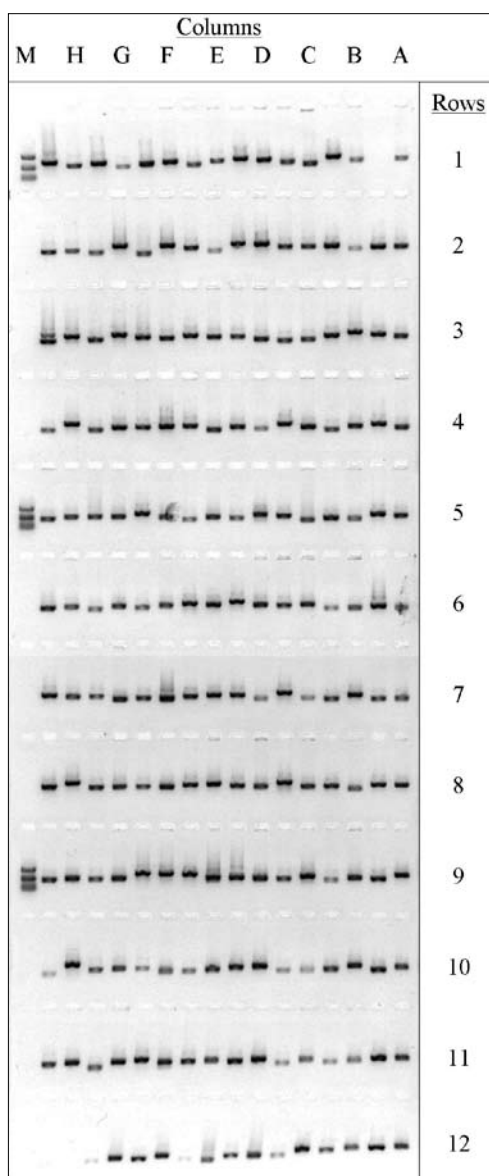


Figure 3
PCR amplification of 192 cowpea drought expression cDNA clones from library plates 17 and 18 using the SP6 and T7 primers. M: ElectroFast marker (200, 500 and 1000bp); columns H–A: columns H to A on the 96-well culture plate 18, respectively; rows 1–12: rows 1 to 12 on the 96-well culture plate, respectively.

analysis of library plates 17 and 18 indicated that PCR amplification was successful for these plates and that the clones contain single inserts (Fig. 3). Purified products of plates 17 and 18 were loaded into alternating wells, since the stretch gel has a capacity for two 96-well PCR plates. All, except 317 clones from the total library, yielded PCR products with sizes between 200 bp and 1 kb (results not shown).

Future work

Now that the cowpea drought-response cDNA library has been generated and the fragments amplified by PCR, a number of steps will follow towards achieving the long term goal of identifying drought-tolerance genes.

Micro-array analysis will be performed on the library's clones to verify their differential expression in the two populations. This is a cutting edge technology currently performed at only two academic institutions in South Africa (The University of Pretoria and the University of Cape Town). It facilitates the investigation of expression levels of high numbers of genes or clones simultaneously. The amplified cDNA fragments will be spotted onto a micro-array slide using a high-density robotic arrayer and hybridised to labelled cDNA or RNA from different sources in order to compare their expression levels.

Expression patterns of interesting genes that emanate from the micro-array study will be investigated further by employing real-time reverse-transcription PCR on RNA samples extracted from differentially drought-treated cowpea plants.

Future objectives of this study include obtaining the full-length sequences of promising genes by genome walking, which will enable the genetic engineering of crops grown by resource-poor farmers for increased drought tolerance. Subsequent testing will confirm if the inserted gene(s) is effective in improving the drought tolerance of the chosen crop, leading to improved food security in water-stressed areas.

Conclusion

The first step in identifying and isolating the genes conferring drought tolerance in cowpea has been successfully completed. This was the construction of a cDNA library enriched for differentially expressed transcripts. The completed cowpea cDNA library will be a valuable resource in the investigations of drought tolerance, as well as other characteristics, of cowpea. Micro-array studies will follow to identify clones of importance during drought tolerance.

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