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Identification of a set of genes from genotypes of common bean tolerant and susceptible to water stress for a macroarray-based selection strategy

G.M. GUTIERREZ-BENICIO[1], J.G. RAMIREZ-PIMENTEL[1], J.A. ACOSTA-GALLEGOS[2],
C.L. AGUIRRE-MANCILLA[1], J.C. RAYA-PEREZ[1], A.P. RODRIGUEZ-VERA[2],
and V. MONTERO-TAVERA[2]*

Tecnológico Nacional de México, Instituto Tecnológico de Roque. Celaya, 38110, México[1]
Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Celaya, 38110, México[2]

Abstract

Globally, drought is the main factor that reduces common bean yield. For this reason, breeding alternatives, such as molecular marker-assisted selection, that focus on various functional genes directly involved in the response to water stress, such as those encoding late embryogenesis abundant (LEA), early response to dehydration (ERD), and dehydrin proteins, have been implemented. The aim of this study was to identify differentially expressed genes of *Phaseolus vulgaris* in drought-tolerant cultivars Pinto Saltillo (PS) and Pinto Villa (PV), and drought-susceptible cultivars Bayo Madero (BM) and Canario 60 (C60) in vegetative and reproductive stages. Relative water content (RWC) in leaf tissue was measured. Twenty-eight *P. vulgaris* genes obtained from GenBank and from a subtractive suppressive library from the PS cultivar were analysed, and their expression profiles were examined by reverse transcription polymerase chain reaction (RT-PCR). Then, cDNA arrays were developed and hybridised to confirm expression which was finally validated by quantitative PCR (qPCR). The usefulness of the identified genes as selection criteria for the tolerance of different genotypes to drought was examined using cDNA arrays. Expression of 21 genes was induced by drought. The cDNA arrays confirmed that expression of 19 of these genes increased in the vegetative stage upon exposure to the drought, and a higher expression was observed in the reproductive stage compared with vegetative stage V4. Only five genes induced by the drought were found to have a lower expression in the susceptible cultivars compared with the tolerant ones. During recovery after the drought in the reproductive stage, 13 of the 21 induced genes remained transcriptionally active including *LEA3* and *dehydrin*. The RWC during the drought in vegetative stage V4 decreased by about 55 % in all cultivars, but at the onset of flowering, it increased to 80 % in PV and PS. In contrast, in the susceptible cultivars, it remained at 55 %. Using qPCR validation, expression induction was confirmed in the drought-tolerant cultivars. *Polyubiquitin2*, *LEA3*, *LEA4*, and *dehydrin* were useful genes for selecting drought-tolerant genotypes under field conditions.

Additional key words: dehydrin, expression patterns, LEA, qPCR, relative water content.

Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the important crops because of its nutritional and nutraceutical qualities (Guzman-Maldonado *et al.* 2002) but a yield is severely affected by drought (Moreno-Garcia *et al.* 2012). Drought is a multidimensional stress that affects plants at various levels of organization (Acosta *et al.* 1999). A water deficit during the reproductive phase decreases bean yield more drastically than a water deficit during the vegetative phase (Acosta-Diaz *et al.* 2004, Aguilar-Benitez *et al.* 2012). Traditional breeding has been applied to increase bean production under drought stress (Ayele 1994, Kornegay *et al.* 1997) leading to development of common bean cultivars tolerant to drought, including Pinto Villa (PV) and Pinto Saltillo (PS) obtained by Acosta *et al.* (1999) and Sánchez-Valdez *et al.* (2004), respectively. However,

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Abbreviations: BCIP - 5-bromo-4-chloro-3-indolyl phosphate p-toluidine; CAB proteins - chlorophyll *a* and *b* binding proteins; ERD - early response to dehydration; GMFL - *Glycine max* full-length; LEA - late embryogenesis abundant; MIPS - myo-inositol-1-phosphate synthase; NCED - 9-cis-epoxycarotenoid dioxygenase; RT-qPCR - reverse transcription - quantitative polymerase chain reaction; RWC - relative water content; ZEP - zeaxanthin epoxidase.

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* Corresponding author; fax: (+52) 4616115323; e-mail: montero.victor@inifap.gob.mx

traditional breeding is a long process, and genotype-environment interactions make rapid advances difficult (Rosas *et al.* 2003). An alternative method is molecular marker-assisted selection which allows identification of genotypes that are tolerant to water deficit in the early stages of plant development (Tanksley *et al.* 1989). These molecular markers include expression markers which provide information on the functions of various genes under different stresses, environmental conditions, or upon different cellular events. Overexpression of several genes in response to water deficit has been observed in a variety of plant species. In *Arabidopsis thaliana* and *P. vulgaris*, 9-cis-epoxycarotenoid dioxygenase (*NCED*) gene induction has been observed (Melhorn *et al.* 2008), and the zeaxanthin epoxidase (*ZEP*) gene has been identified in *Nicotiana plumbaginifolia*, *Arabidopsis thaliana*, and *Solanum lycopersicum* (Bittner *et al.* 2001). These genes are related to synthesis of abscisic acid, a growth regulator that mediates a response to drought stress. A group of induced genes encoding late embryogenesis abundant (LEA) proteins, which protect membranes from damage by dehydration, have been studied during osmotic stress (Colmenero-Flores *et al.* 1999). Another pair of genes, *RD29A* and *RD29B*, have mainly been studied in *Arabidopsis thaliana* and are related to synthesis of transcription factors (Liu *et al.* 1998, Nakashima *et al.* 2000). In the bean cv. PV, overexpression of 28 genes, associated with drought tolerance, that encode LEA proteins, transcription factors, photosynthesis-related proteins, and protein stabilization-related proteins has been reported (Barrera-Figueroa *et al.* 2007). In vascular tissue of cv. PS, three genes that encode proteins related to mobilization signals in phloem and to the integrity of the vascular tissue under water stress have been identified (Montero-Tavera *et al.* 2008). The aim of this study was to identify differentially expressed genes in drought-tolerant cvs. PS and PV and drought-susceptible cvs. Bayo Madero (BM) and Canario 60 (C60) under water stress in vegetative and reproductive stages.

Materials and methods

Bean (*Phaseolus vulgaris* L.) seeds from two drought-tolerant cultivars PV and PS, and two susceptible cultivars BM and C60 were germinated on moistened filter paper in a growth chamber (*Conviron*™) at 27 °C, and after 5 d, the seedlings were transplanted into *Sunshine*® substrate mix *No. 3* (*SunGro*®, USA) and transferred to a greenhouse (a 12-h photoperiod, an irradiance of 570 μmol m^{-2} s^{-1}, day/night temperatures of 28/15 °C and an air humidity of 50 - 64 %). For each bean genotype, 4 groups of 10 plants were established. A control group was irrigated throughout the entire plant life cycle. In the second group of plants, irrigation was suspended for 5 d during vegetative stage V4 (White 1988), and in the third group of plants, irrigation was suspended for 5 d at the onset of flowering (the reproductive stage). In the fourth group of plants, irrigation was suspended for 5 d twice during V4 stage and the onset of flowering. On the fifth day of water suspension, the plants showed wilting foliage and mostly folded leaves. After that, the plants were irrigated and allowed to recover for 10 d. Leaves were collected after the water stress and after the recovery and stored at -80 °C for further processing.

Relative water content (RWC) of leaves was determined as described in Woo *et al.* (2008). Leaf tissue samples (15 mm in diameter) were collected for each treatment, and fresh mass (FM) was recorded. Then, the samples were saturated with water at room temperature for 18 h, and saturated mass (SM) was recorded. Finally, the samples were dried in an oven at 80 °C for 24 h, and dry mass (DM) was determined. To calculate the RWC, the following formula was used:

$$\text{RWC [\%]} = [(\text{FM} - \text{DM})/(\text{SM} - \text{DM})] \times 100.$$

The total RNA was extracted from leaf tissue as described by Logemann *et al.* (1987). The RNA concentration was standardised to 0.4 g dm^{-3}, and mixtures that included material from 10 plants for each of the experimental conditions were examined. The quality of RNA was assessed on a 1.5 % (m/v) agarose gel under denaturing conditions. A cDNA was synthesised by real-time polymerase chain reaction (PCR), and each reaction mixture consisted of 0.001 cm^3 of 3' *SMART*™ *CDS* primer IIA, 0.002 cm^3 of RNA (0.8 μg), 0.001 cm^3 of *SMART*™ IIA primer, 0.001 cm^3 of a deoxynucleotide solution mix (0.01 M), and 0.006 cm^3 of water. This mixture was incubated at 65 °C for 5 min, and then 0.005 cm^3 of a 5× reaction buffer (*Invitrogen*, Carlsbad, USA), 0.002 cm^3 of dithiotreitol (0.1 M) and 0.0005 cm^3 of *Super Script II* enzyme were added. The sample was incubated at 42 °C for 50 min, and the reaction was stopped immediately by incubation at 70 °C for 15 min. The cDNA amplification from the four bean cultivars and each treatment was standardised to the *26S* ribosomal housekeeping gene (Ruiz-Nieto *et al*., 2015). To study expression profiles associated with drought-related genes, eight pairs of primers that had been previously constructed in our laboratory and had been designed from a suppressive subtractive library from cv. PS under drought conditions were used in addition to 13 primer pairs designed from a Gene bank of cv. PV (Barrera-Figueroa *et al*., 2007, Table 1 Suppl). Each PCR mixture contained 0.012 cm^3 of water, 0.001 cm^3 of a deoxynucleotide solution mix (0.01 M), 0.002 cm^3 of a *Taq* buffer (*Invitrogen*), 0.0008 cm^3 of $MgCl_2$, 0.001 cm^3 of each primer (1 μM), 0.0002 cm^3 of *Taq* polymerase (*Invitrogen*), and 0.002 cm^3 of cDNA (500 ng). To visualize the amplified genes, a 1.5 % (m/v) agarose gel was used, and the bands were observed using 0.1 % (m/v) ethidium bromide staining.

The gene fragments amplified by RT-PCR were cut from the agarose gel, purified with a *QIAquick* kit (*Qiagen*, Germany), ligated into the cloning vector *pGEM-T-Easy* (*Promega*, Madison, USA), and transformed into cells of competent *E. coli* strains DH-5α and JM 107 by heat shock (Sambrook and Russell, 2006). The transformed cells were plated on a Lourie-Brot agar growth medium supplemented with 0.1 g dm^{-3} ampicillin, 50 g dm^{-3} 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and 23.8 g dm^{-3} isopropyl β-D-1-thio-galactopyranoside (IPTG) (*Sigma*, St. Louis, USA) and incubated at 37 °C for 18 h. The positive clones were selected and propagated in a Lourie-Brot liquid medium. The plasmid DNA was purified using the *Quick* minipreps method (Zhovic and Yong 1990), and the resulting DNA was denatured and used for analysis of cDNA arrays.

Duplicates of 0.750 µg of the plasmid DNA containing selected gene probes were printed onto *Hybond-N*$^+$ nylon membranes (*Amersham Biosciences*, Uppsala, Sweden). A 26S ribosomal gene fragment was used as positive control for hybridisation, and water and the plasmid without an insert were used as negative control. For all the treatments, the double stranded (ds) cDNA was synthesised with an advantage cDNA polymerase enzyme mix (*Clontech*, Palo Alto, USA) and subsequently purified with a *QIAquick* kit. A total of 1 750 µg of each purified ds cDNA was used as probe. A total of 28 probes were synthesised, corresponding to each of the treatments. The probes were labelled with biotin-11-dUTP by random priming (Feinberg and Vogelstein 1983) with a biotin *DecaLabel*™ DNA labelling kit (*Fermentas*, Lithuania). Hybridization was detected with a biotin chromogenic detection kit (*Fermentas*) using streptavidin coupled to alkaline phosphatase and nitroblue tetrazolium - 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine as chromogenic substrate.

Densitometric image analysis of cDNA array membranes was performed with the *TotalLabQuant TL120 v. 2008* software to identify differentially expressed genes. The spots in the membrane images were normalised for brightness and contrast with respect to the signal of the *26S* ribosomal gene, and then the intensity of the signal of each gene was measured. For densitometric analysis, the signal intensity of the 26S gene was normalised in each of the bean cultivars, and the standard deviation was calculated. The threshold of gene expression was estimated with respect to the control for each of the identified genes and compared to the control for each of the identified genes.

To confirm the expression profiles revealed by RT-PCR and cDNA arrays, six of the genes identified as differentially expressed were evaluated by quantitative PCR (qPCR) using a *Step One* real-time PCR system (*Applied Biosystems*). The genes examined encoded *Glycine max* cDNA, clone: GMFL01-08-H07 (GMFL), polyubiquitin2, α-carboxyl-transferase, sulphur-rich proteins, an elongation factor, and LEA3, and a selection criterion for evaluation was high transcriptions in cvs. PS and PV. The reaction mixtures contained 0.0125 cm^3 of *Master Mix* (*Thermo Scientific*, USA) with a fluorophore *SYBR Green/ROX*, 0.0015 cm^3 of appropriate primers (0.005 M), 0.01 cm^3 of water, and 0.001 cm^3 of a single-stranded cDNA (0.05 g dm^{-3}). To determine amplification efficiency, calibration curves were constructed from five serial dilutions (1:10) of the cDNA (a 0.4 µg cm^{-3} initial concentration) with three replications. The experimental conditions were standardised using a primer for the *26S* gene. The results were analysed with the comparative C_T method using a mathematical model proposed by Pfaffl (2002) and the *REST 2009 v. 2.0.13* software (*Qiagen*).

To determine functionality of the cDNA array, 36 different bean genotypes were subjected to drought at the beginning of the flowering stage, and leaf tissue was sampled after the drought and re-irrigation treatments. In the field, 18 cultivars characterised as drought-tolerant were planted. Six lines were crosses between the genotypes Pinto Durango/PS, six were black bean cultivars, and other six were red bean cultivars. Sampling was performed when the water content of the soil was 23 %. In the greenhouse, an additional 18 cultivars with unknown drought tolerance were planted. Lines, crosses, and cultivars developed by *INIFAP* (Guanajuato, Mexico) were included among these, and drought treatment was maintained until the water content of the soil was 7 %. Four drought response genes, *LEA3*, *LEA4*, *DEHYDRIN*, and *POLYUBIQUITIN2*, were used as hybridization probes. The probe and array were developed as described above, and the total cDNA from each genotype was printed in duplicate on nylon membranes. Subsequently, densitometry analysis was performed, and the ratio of the expressions during the drought treatment and in the irrigated control (drought/irrigation) was calculated. Values of the ratio greater than 1.0 were considered as induction of gene expression.

Results

The analysis of RWC in plant leaves (Fig. 1) shows that the control plants (under irrigation) of all the cultivars maintained the RWC at approximately 80 %. In the plants subjected to the drought stress during V4 stage, the RWC decreased to approximately 55 % and returned back to 80 % after the recovery. When the second water stress was applied in the reproductive stage, the RWC in the tolerant cvs. PV and PS remained similar to that of the control, whereas in the susceptible cvs. C60 and BM decreased to 50 %. When the PV and PS plants received the single stress during the reproductive stage, their RWC decreased near to 60 %. These results demonstrate that the plants subjected to the water stress during the vegetative stage activated response mechanisms to reduce damage during further water stress.

Twenty-one genes showed differential expressions during the drought treatment based on the RT-PCR (Fig. 1 Suppl.). The activation of transcription was mainly identified in the tolerant cvs. PV and PS in which the induction of genes was identified after the drought in vegetative stage V4 although some genes remained active also after the recovery. In the reproductive stage, gene expressions observed after the second stress were similar to those observed in plants that received only the single stress. Most of the genes induced in the reproductive stage (except PS and BM) remained active after the recovery. In the susceptible cvs. C60 and BM, a slight induction of gene expression was observed.

The densitometric analysis of cDNA arrays revealed differential expression between the tolerant and susceptible cultivars (Tables 1 and 2). The tolerant cvs. PV and PS showed the highest number of induced genes under the water stress, whereas the susceptible cvs. C60 and BM showed repression of most identified genes. The highest overall expressions were identified in PV (1.42- to 22.7-fold) and PS (1.02- to 19.4-fold), whereas in the susceptible cultivars, the expressions ranged between 1.03- and 3.36-fold. The drought stress during the reproductive stage induced a higher expression than in V4 stage especially in the tolerant plants. A higher

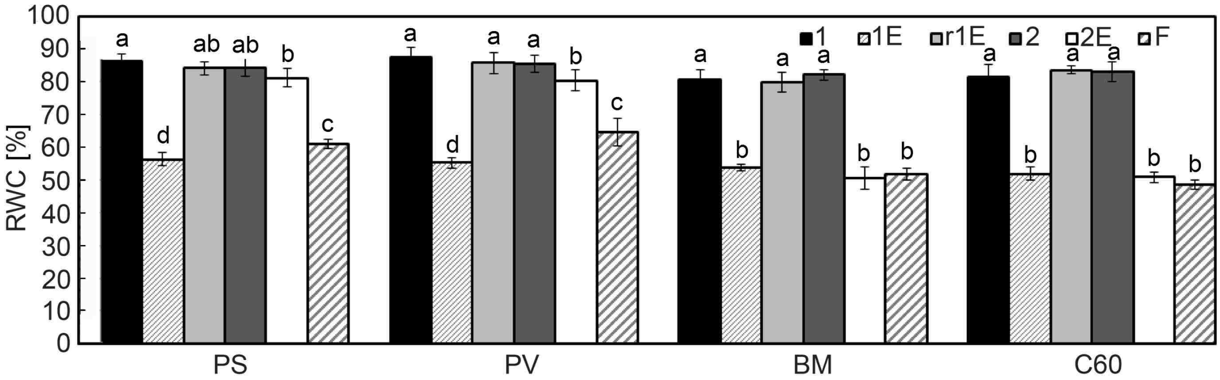

Fig. 1. Leaf relative water content (RWC) in tolerant cvs. PS and PV and susceptible cvs. BM and C60. 1 - control in the vegetative stage, 1E - first water stress at vegetative stage V4, r1E - recovery after first water stress, 2 - control in the reproductive stage, 2E - second water stress at reproductive stage, F - one stress in the reproductive stage. Means with the same letter are not significantly different according to Tukey ($P > 0.05$).

Table 1. Differential gene expression in response to drought in the tolerant cultivars as revealed by cDNA arrays. Ratio of the first stress to the control (1E/C) and recovery (R1E/C); ratio of the second stress to the control (2E/C) and recovery (R2E/C); ratio of a single stress at the beginning of flowering to the control (EF/C) and recovery (RF/C). Induced genes (*dark grey*), constitutive genes (*white*), and repressed genes (*light grey*).

Encoded proteins	Pinto Saltillo				Pinto Villa					
	1E/C	R1E/C	2E/C	EF/C	1E/C	R1E/C	2E/C	R2E/C	EF/C	RF/C
Hypothetical protein	1.44	1.34	0.96	3.44	8.00	4.35	3.16	0.87	0.91	0.84
Clone GMFL 01-08-H07	2.68	0.10	4.31	6.99	8.60	1.10	3.40	0.94	1.39	1.95
Protein ERD	1.71	1.54	5.09	4.83	2.60	1.56	2.08	0.81	1.20	0.32
Sulphur-rich proteins	3.17	1.21	4.39	5.51	4.77	0.48	5.21	0.30	1.78	1.46
Photosystem II proteins	0.54	0.63	2.02	2.83	4.56	2.26	6.50	3.33	2.95	2.64
Peptidase family M13	1.13	0.11	1.23	3.85	8.76	6.19	5.30	0.37	1.30	1.9
LEA3	0.92	1.03	11.2	17.6	4.83	2.68	13.1	1.8	3.41	0.62
Microsatellite SSR-IAC 105	1.33	5.47	1.75	2.32	2.97	1.51	0.97	0.37	0.48	1.82
Type 2 metallothionein	3.99	0.83	1.08	0.97	2.47	2.40	13.5	1.45	0.96	1.34
Polyubiquitin2	2.15	2.30	1.35	11.5	1.70	1.15	0.45	0.03	0.06	0.16
Elongation factor 1A	3.05	2.18	3.07	2.32	4.48	1.72	2.04	0.06	0.53	0.38
Myo-inositol-1-phosphate synthase	1.51	1.18	1.97	1.46	1.42	0.60	2.76	0.45	0.11	0.68
Hypothetical protein CAB71113	1.25	0.51	0.16	1.55	3.54	1.87	0.93	0.25	1.93	0.54
Periplasmic-cytochrome C	5.82	0.49	4.23	6.22	0.43	0.31	1.10	0.77	1.68	0.37
Dehydrin	1.02	1.23	2.04	3.51	2.65	1.35	3.09	0.81	2.55	1.19
Transmembrane a.a. transporter protein	4.63	5.43	0.64	1.63	1.9	0.61	5.09	0.87	0.24	6.06
LEA4	1.13	0.16	16.6	10.2	2.01	0.95	5.10	1.28	1.12	0.28
Glycosylation enzyme	0.60	0.23	6.94	12.9	3.82	1.30	4.53	2.39	1.76	1.92
Clone 169	2.3	0.73	1.33	5.54	0.67	2.23	4.43	1.03	1.49	0.99
α-Carboxyltransferase	1.05	1.11	1.06	2.91	2.97	1.25	0.46	0.14	0.25	0.29
Clone 033	0.94	0.83	9.11	19.4	3.44	1.22	22.7	3.40	0.81	3.00

Table 2. Differential gene expression in response to drought in the susceptible cultivars as revealed by cDNA arrays. The ratio of the first stress to the control (1E/C) and recovery (R1E/C); ratio of the second stress to the control (2E/C) and recovery (R2E/C); ratio of a single stress at the beginning of flowering to the control (EF/C) and recovery (RF/C). Induced genes (*dark grey*), constitutive genes (*white*), and repressed genes (*light grey*).

Encoded proteins	Bayo Madero				Canario 60					
	1E/C	R1E/C	2E/C	EF/C	1E/C	R1E/C	2E/C	R2E/C	EF/C	RF/C
Hypothetical protein	1.03	0.95	0.22	0.29	3.80	9.94	0.32	0.69	0.12	0.69
Clone GMFL 01-08-H07	0.84	0.63	0.36	0.14	0.63	0.19	0.41	0.53	0.19	0.53
Protein ERD	1.43	0.43	0.32	0.14	0.48	1.49	0.17	0.68	0.06	0.68
Sulphur-rich proteins	0.0	0.41	0.3	0.31	0.62	0.37	1.37	0.76	0.04	0.76
Photosystem II proteins	0.94	0.88	0.21	0.32	0.52	0.41	0.47	0.66	0.10	0.66
Peptidase family M13	1.54	1.05	0.19	0.31	0.34	0.93	0.16	0.36	0.07	0.36
LEA3	1.00	1.43	0.68	0.80	0.37	0.43	0.33	0.18	0.21	0.18
Microsatellite SSR-IAC 105	0.96	1.24	0.66	0.80	0.47	0.44	0.18	0.73	0.27	0.73
Type 2 metallothionein	0.96	2.53	0.54	0.68	1.24	0.38	0.19	0.28	0.26	0.28
Polyubiquitin2	0.93	2.21	0.78	0.90	0.54	0.44	0.25	0.28	0.54	0.28
Elongation factor 1A	0.54	0.77	0.67	0.56	0.61	0.45	1.69	0.84	0.99	0.84
Myo-inositol-1-phosphate synthase	1.38	1.86	1.25	0.40	1.36	3.36	0.31	0.61	0.11	0.61
Hypothetical protein CAB71113	1.34	6.62	0.13	0.38	1.03	0.46	0.40	0.65	0.06	0.87
Periplasmic cytochrome C	0.50	2.00	0.56	2.12	1.48	0.34	0.24	0.87	0.08	1.59
Dehydrin	0.59	0.41	0.53	0.85	0.36	0.80	0.17	0.68	0.13	0.65
Transmembrane a.a. transporter protein	0.33	0.78	0.11	0.04	0.06	0.25	0.21	0.14	0.08	0.14
LEA4	0.66	0.83	1.39	1.43	0.47	0.64	0.22	0.48	0.06	0.21
Glycosylation enzyme	2.27	1.08	0.25	0.42	0.48	0.66	0.12	0.51	0.13	0.14
Clone 169	1.25	0.67	0.07	0.29	0.47	0.51	0.11	0.56	0.06	0.26
α-Carboxyltransferase	4.83	0.89	0.46	1.32	0.39	0.82	0.32	0.76	0.12	0.91
Clone 033	1.06	0.19	1.07	0.43	0.53	1.89	0.40	0.82	0.28	1.10

Table 3. Differential expression (drought/irrigation ratio) as assessed by qPCR in different cultivars (PS, PV, BM, and C60) after the first water stress (1E) and after the second water stress (2E). Means ± SD; ** - highly significant differences ($P < 0.01$), * - significant differences ($P < 0.05$). The *arrows* indicate a greater expression than the control.

Encoded proteins	PS1E	PV1E	BM1E	C601E
Clone GMFL 01-08-H07	↑ 3.46 ± 0.23 **	↑ 3.44 ± 0.25**	↑ 2.50 ± 0.26*	↑ 13.80 ± 0.19*
Polyubiquitin2	↑ 10.70 ± 0.35*	↑ 355.44 ± 0.25**	0.24 ± 0.44**	0.38 ± 0.34*
α-Carboxyltransferase	↑ 46.84 ± 0.28**	↑ 5.40 ± 0.16*	↑ 3.56 ± 0.51*	1.00 ± 0.17
	PS2E	PV2E	BM2E	C602E
Sulphur-rich proteins	↑ 119.00 ± 0.11**	↑ 135.70 ± 0.14**	↑ 24.12 ± 0.12*	↑ 24.99 ± 0.25*
Elongation factor 1A	↑ 128.45 ± 0.19**	↑ 16.93 ± 0.18**	0.22 ± 0.17	0.23 ± 0.27*
LEA3	↑ 34.89 ± 0.09*	↑ 100.54 ± 0.18**	↑ 9.07 ± 0.27*	↑ 17.60 ± 0.11*

expression during the reproductive stage was observed in cv. PV in the double water stress treatment compared to the single stress-treated plants, whereas cv. PS showed an opposite effect.

Regarding the tolerant cultivars, 17 genes were induced in PS and 19 genes in PV during the first stress (in V4); those genes were associated with the expression of early response to dehydration (ERD) proteins, sulphur-rich proteins, photosystem II proteins, LEA3, polyubiquitin2, myo-inositol-1-phosphate synthase, dehydrin, LEA4, and α-carboxyltransferase. During the second stress (the reproductive stage), 16 genes were induced in PV and 14 genes were induced in PS, whereas when the single stress was applied in the reproductive stage, PS had a higher number of induced genes than PV (20 and 7 genes, respectively); at this stage, the induced genes encoded LEA3, dehydrin, LEA4, photosystem II proteins, and ERD. In the susceptible ciltivars in the vegetative stage, four genes were activated in BM and five in C60. During the second stress, only two genes were induced in C60. After the single stress during the reproductive stage, the expressions of three genes were induced in BM, whereas none were activated in C60. During the recovery, several genes that were activated during the first water stress remained active. In PS, there were nine genes that remained active. In PV, 13 genes maintained the same expression. In C60, two genes maintained the same expression, but in BM, no genes remained active.

Table 4. Drought-induced gene expression in bean genotypes grown under field conditions as evaluated by cDNA array. Soil moisture was decreased to 23 %. The ratio of expression during drought treatment and irrigated control.

Genotypes	*LEA3*	*POLYUBIQUITIN2*	*DEHYDRIN*	*LEA4*	Mean
Pt. Dgo/Pt. Sal-2-3	0.499	0.453	0.385	0.698	0.508
Pt. Dgo/Pt. Sal-6-7	0.916	0.840	0.406	1.000	0.790
Pt. Dgo/Pt. Sal-6-6	0.989	1.726	0.912	0.943	1.142
Pt. Dgo/Pt. Sal-11-3	1.165	2.142	0.810	0.993	1.277
Pt. Dgo/Pt. Sal-4-4	4.284	1.604	0.906	0.835	1.907
Pt. Dgo/Pt. Sal-11-2	0.266	1.130	0.702	0.578	0.669
SCR13	0.958	0.902	0.447	0.513	0.705
SER 83	0.987	5.228	1.375	1.155	2.186
SER 118	1.595	1.116	1.278	0.986	1.243
SCR 11	3.778	1.993	1.531	0.986	2.072
SCR 18	1.957	1.461	0.927	0.882	1.306
INTA SEQUIA	2.909	1.623	1.116	0.991	1.659
SCN 7	0.840	1.136	0.873	1.253	1.025
SEN 56	0.647	0.656	0.809	0.839	0.737
SEN 26	0.726	0.247	0.619	0.797	0.597
SEN 70	0.412	0.416	0.522	0.670	0.505
SEN 44	0.918	0.481	1.963	2.427	1.447
ELS 15 55	0.554	1.286	1.128	0.791	0.939

Table 5. Drought-induced gene expression in bean genotypes grown under greenhouse conditions as evaluated by cDNA array. Soil moisture was decreased to 7 %. The ratio of expression during drought treatment and irrigated control.

Genotypes	*LEA3*	*POLYUBIQUITIN2*	*DEHYDRIN*	*LEA4*	Mean
Pv/ps -11	0.354	1.111	0.651	0.656	0.693
SER 83	0.210	1.200	0.880	0.797	0.771
Rosa la bufa	0.813	1.134	0.770	0.427	0.786
G4523	0.824	0.841	0.907	1.061	0.908
SER 18	1.075	0.987	0.794	1.744	1.150
PV	0.562	0.930	1.216	0.593	0.825
F.J. Leon	0.755	2.347	1.143	1.193	1.359
SCR 6	0.572	0.871	0.778	1.021	0.810
F.J. Dalia	0.250	0.837	0.266	0.237	0.397
Bayo Madero	0.742	1.117	0.919	0.943	0.930
SCR 16	0.377	1.032	0.422	0.791	0.655
F.M. Dolores	0.355	1.361	0.738	0.889	0.835
Pinto Salinas	0.716	1.065	0.685	0.701	0.791
Pt. San Luis	0.500	0.983	0.892	0.684	0.764
Pv/Ps -8	1.030	0.491	0.736	0.730	0.746
Pv/Ps -281	0.812	1.035	0.524	0.489	0.715
Pt. San Rafael	0.719	1.283	0.896	0.432	0.832
Pt. Raramuri	1.343	0.834	0.943	0.567	0.921

From the genes activated during the reproductive stage and examined during the recovery, PV was the only cultivar in which six activated genes maintained the same expression. The genes encoding photosystem II proteins, LEA3, a glycosylation enzyme, polyubiquitin2, an ERD protein, sulphur-rich proteins, elongation factor 1A, and dehydrin maintained the same expression from V4 to the reproductive stage and throughout the recovery (Table 2 Suppl.).

The qPCR results confirm that the six selected genes were differently expressed among the tolerant and susceptible cultivars in response to the drought (Table 3). In cvs. BM and C60, four genes showed a lower expression than in cvs. PV and PS; in contrast, GMFL was expressed at a higher level in C60 than in BM, PV, and PS. However, the expressions differed among the three methods used, and higher levels were observed using qPCR. For the susceptible cultivars, three genes, for which no expression was observed by RT-PCR, were detected by qPCR although the expressions of these genes were low.

Of the 36 bean genotypes evaluated with cDNA array, 12 genotypes (greenhouse and field conditions) were found to tolerate the drought. From the genotypes established in the field (Table 4), red genotypes were highlighted in selection, and five of the six evaluated showed an induction of expression for three genes. The lines obtained by crossing Pinto genotypes were also selected as well as two black genotypes which showed expression of two genes. Of the greenhouse grown genotypes, two showed an increase in expression of two genes (Table 5). In contrast, the genotype F.J. León showed induction of three genes. The cDNA array used in this research to select drought tolerant genotypes allowed to identify bean cultivars with this feature. Most drought-responsive genes were identified in bean genotypes produced under field conditions with a history of conventional breeding tolerance to water stress; on the other hand, a low number of genes in response to drought were identified in genotypes produced under greenhouse conditions with no history of breeding to this stress; this confirms the functionality of the cDNA array proposed here to be incorporated into a breeding programme of molecular marker-assisted selection.

Discussion

Previous investigations have reported changes in gene expression in response to water stress, including changes in expression of genes involved in production of enzymes for synthesis of osmolytes and proteins with protective functions such as LEA proteins, antioxidant enzymes, and transcription factors (Moreno 2009). The genes expressed under the water stress reported in this paper include those associated with synthesis of various proteins such as LEA3, LEA4, and dehydrin (LEA2). These proteins are essential for a drought response because they provide a hydrophilic microenvironment due to their random coil structure; thus, they can substitute for water and help to maintain the structures of various proteins as well as membrane integrity (Battaglia *et al.* 2008). Therefore, they have been widely studied in various organisms, and transcripts have been identified in vascular and non-vascular plants in response to dehydration (Salmi *et al.* 2005). Another group of proteins involved in water stress are ERD proteins which protect the cell membrane against damage by dehydration (Barrera-Figueroa *et al.* 2007, Alves *et al.* 2011). The ERD proteins, such as ERD4, are rapidly induced by water deficit and have been characterized mainly in maize, *Arabidopsis*, and rice (Rai *et al.* 2012). We identified transcriptional induction under the drought stress treatment at an early stage of development. Additionally, within the set of induced genes, we identified several genes with a possible role in photosynthesis (Jaramillo-Giraldo *et al.* 2009). During drought stress, there is an imbalance of ionic homeostasis that results in an increased content of Ca^{2+} which acts asmessenger in signalling pathways to activate transcription of genes responsive to drought. Millar *et al.* (1994) reported that an increasing content of cytosolic Ca^{2+} induces transcription of genes encoding structural photosystem II proteins and chlorophyll *a* and *b* binding (CAB) proteins. In our study, we identified photosystem II proteins, CABs, and a periplasmic cytochrome. Transcription of genes related to photosynthetic processes is critical because their joint action could ensure photosynthetic efficiency in response to dehydration. Four genes related to metabolism were identified. The first of them was related to biosynthesis of sulphur-rich proteins with possible involvement in synthesis of storage proteins in seeds of common bean (Yin *et a*l. 2011). Further, albumins with sulphur-containing amino acids have been identified in different cultivars of common bean (Raya-Perez *et al.* 2014). The second gene associated with metabolism encodes a protein related to carboxyltransferase which takes part in fatty acid synthesis, specifically with acetyl-CoA carboxylase (Natsumi *et al.* 2013). Other induced genes belong to the amino acid transporter superfamily and include amino acid transporter family 1 (ATF1) and solute carrier 38 (SLC38) (Wipf *et al.* 2002). Another gene from this group is elongation factor 1A which is involved in protein synthesis and has been isolated and characterized. In rice, the same genes (*refa1*, *refa2*, *refa3*, *refa4*) are expressed in different tissues and stages of plant growth (Shin-ichiro *et al.* 1998). These four genes were identified after drought treatments and emerged as help to cellular metabolism to prevent structural alterations and lipid oxidation that can occur due to water deficit. Furthermore, metallothionein2 is a typical class of plant proteins that chelates heavy metals such as cadmium and copper, and this type of protein has been observed in rice and *Arabidopsis* (Zhou and Goldsbrough 1995, Zhou *et al.* 2006). *METALLOTHIONEIN* genes have been characterized in *Jatropha curcas* (Shalini *et al.* 2014), and the data suggest that induction of these genes during drought might contribute to detoxification mechanisms. Myo-inositol-1-phosphate synthase (MIPS), which is involved in biosynthesis of myo-inositol, was identified and is an important second messenger in water stress signal transduction (Irvine and Schell 2001). The MIPS has been identified to show differential expressions in *Phaseolus vulgaris* (Johnson and Wang 1996), and Abid *et al.* (2009) reported the identification of 60 MIPS genes in different organisms involved in tolerance to biotic and abiotic stresses. Various proteins, both structural and functional, are affected by water deficit, and therefore, mechanisms, such as glycosylation and ubiquitination, are required to counteract this effect. In a list of drought-induced genes, the gene *POLYUBIQUITIN2* encodes a protein that marks proteins for degradation or recycling (Welchman *et al.* 2005), and glycosylation enzymes play a substantial role in maintaining cellular homeostasis by modifying various molecules, primarily proteins (Wang and Hou 2009). These genes might be involved in recovery of cell damage due to water stress. In addition to the set of the identified genes, hypothetical genes GMFL, microsatellite SSR-IAC 105 of *Phaseolus vulgaris*, peptidase family M13, clone 169, and clone 033 were identified. Although they do not seem to have a direct relationship with the water deficit response, their expressions were altered upon the drought treatment.

In this study, the identification of several genes induced by water stress in widely recognized cultivars that are tolerant to drought was crucial; moreover, the induction of expression in early development during the vegetative phase and the continuous expression of some transcripts after the plants recovered were vital information. Additionally, an understanding of the expression of these genes during the reproductive stage, which is sensitive to drought damage, might be helpful for elucidating molecular mechanisms involved in the response to drought. Thus, this set of identified genes is of major agricultural and biotechnological importances. In this paper, the utility of some genes that are directly related to drought response, mainly *LEA3*, *LEA4*, *POLYUBIQUITIN2*, and *DEHYDRIN*, have been verified, and cDNA array was demonstrated as a feasible tool for selection of different drought tolerant bean genotypes under field conditions. Based on this work, these genes should be considered as expression markers in biotechnological applications to identify promising genotypes.

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