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Differential accumulation of mRNAs in drought-tolerant and susceptible common bean cultivars in response to water deficit

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Summary

• The physiological response to drought was measured in two common bean varieties with contrastive susceptibility to drought stress. A subtractive cDNA library was constructed from the two cultivars, *Phaseolus vulgaris* 'Pinto Villa' (tolerant) and 'Carioca' (susceptible).

• 18 cDNAs displayed protein-coding genes associated with drought, cold and oxidative stress, signal transduction, plant defense, chloroplast function and unknown function. A cDNA coding for an aquaporin (AQP) was selected for further analyses.

• The open reading frames (ORFs) of AQPs from 'Pinto Villa' and 'Carioca' were compared and despite their similarity, accumulated differentially in the plant organs, as demonstrated by Northern blot and *in situ* hybridization. A phylogenetic analysis of the deduced amino acid sequence with other AQPs suggested a tonoplast-located protein.

• Under drought conditions, the levels of AQP mRNA from the susceptible cultivar decreased to undetectable levels; by contrast, 'Pinto Villa' mRNA was present and restricted the phloem tissue. This would allow 'Pinto Villa' to maintain vascular tissue functions under drought stress.

Key words: aquaporin (AQP), differential mRNA expression, drought, *Phaseolus vulgaris*, subtractive library.

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Introduction

Globally, 60% of the common bean variety (*Phaseolus vulgaris*) is cultivated under drought conditions, and second to plant diseases, drought is considered the most important in the reduction of crop productivity (Singh, 1995, 2001). Common bean cultivars have been selected for tolerance to drought conditions in semiarid lands, although the mechanisms for this tolerance have not been characterized (Acosta-Gallegos & White, 1995; Schneider *et al.*, 1997). Plants respond to drought through several strategies, some of which involve the maintenance of water balance. This is achieved by the induction of responses at the transcriptional, post-transcriptional, translational and post-translational levels.

Water uptake and allocation is regulated by a family of water channel proteins called aquaporins (AQPs) (Tyerman *et al.*, 2002). The presence of AQPs in the plasma membrane provides a mechanism to regulate the flux of water between cells (Johansson *et al.*, 2000; Luu & Maurel, 2005). It can be considered possible that AQPs play an important role during

adaptation to water deficit. Indeed, both RNA and protein for some AQPs are induced during osmotic shock, and salt and drought stress (Barrieu *et al.*, 1999; Lopez *et al.*, 2003). However, other isoforms of AQPs are downregulated after drought treatment (Yamada *et al.*, 1995; Li *et al.*, 2000). The case of tonoplast AQPs maybe somewhat different; for example, an *Arabidopsis* tonoplast AQP facilitates the transport of ammonium into the vacuole (Loque *et al.*, 2005), suggesting that these proteins may be involved in the transport of different small molecules (Ma *et al.*, 2004). Overexpression of tonoplast AQPs in *Arabidopsis* had beneficial effects on salt-stress tolerance, as indicated by superior growth status and seed germination (Peng *et al.*, 2007).

Tonoplast intrinsic proteins (TIPs) have been found in the tonoplast of the protein storage vacuoles of *P. vulgaris* seeds, facilitating the transport of ions and metabolites between the storage vacuoles and cytoplasm of seed storage tissues (Johnson *et al.*, 1990). A membrane intrinsic protein (MIP) member from *P. vulgaris* has been found in vacuolar membranes of cotyledons and is synthesized during seed maturation.

We report here the physiological characterization of two common bean varieties with contrastive tolerance to drought stress, as well as the identification and functional classification of mRNAs isolated from the drought-tolerant variety Pinto Villa, which comprise genes associated with drought, cold and oxidative stress, signal transduction, plant defense, chloroplast function and unknown sequences. In particular, the differential accumulation of an AQP transcript, presumably from tonoplast, in two common bean cultivars, which display a contrasting phenotype in response to drought, is described. In *P. vulgaris* 'Pinto Villa', which is drought tolerant, this mRNA is accumulated in the vascular cylinder in the root under water deprivation. By contrast, the bean cultivar Carioca, which is drought-susceptible (Acosta-Gallegos & Adams, 1991), displays low levels of this mRNA in the same tissue in drought conditions, distributed in both root vascular cylinder and root endodermis; however, higher levels are observed in leaf blade in normal, but not drought, conditions. These data suggest that 'Pinto Villa' copes with drought stress, among many other mechanisms, by restricting water translocation through peripheral tissues (parenchyma, for example) and encouraging its transport within the vascular system.

Materials and Methods

Plant material

Two common bean (*P. vulgaris* L.) varieties with contrasting tolerance to drought were selected. 'Pinto Villa' belongs to the middle American gene pool of the race Durango, bearing an indeterminate III growth habitat and high tolerance to drought stress in field trials (Acosta-Gallegos *et al.* 1995; Rosales-Serna *et al.*, 2004). 'Pinto Villa' displayed a drought stress index (DSI) of 0.7, which indicates high mean seed yield, low percentage of seed yield reduction and pronounced maturity acceleration under drought stress (Terán & Singh, 2002). 'Carioca', also called 'rainy bean' (da Silva *et al.*, 2005) is the drought-susceptible variety selected in this study, which belongs to the Mesoamerican gene pool, also with an indeterminate III growth habitat. (Acosta-Gallegos & Adams, 1991; Acosta-Gallegos & White, 1995). 'Carioca' displayed a high salt sensitivity which affects its early vegetative growth (Maas & Hoffman, 1977; Bayuelo-Jiménez *et al.*, 2002). Salinity stress can affect plants by drought stress (Welbaum *et al.*, 1990) and/or ion toxicity (Huang & Reddman, 1995). The normal life cycle from germination to flowering under normal irrigation is 80 d for 'Carioca' and 83 d for 'Pinto Villa'. Both Pinto cultivars are genetically compatible, since new lines from the genetic pools Durango and Mesoamerican, to which they belong, have been crossed to obtain drought-tolerant plants with resistance to abiotic stress (Singh, 2001).

Seeds of each cultivar were disinfected with 10% H_2O_2 for 10 min and germinated in humid plastic bags with glass fiber for 5 d. The seedlings were transferred to 50 pots with a capacity of 4 l in volume with peat moss (Sunshine, Mc Calif, CA, USA) as substrate, filling approx. 4/5ths of the total capacity. Four seedlings were planted in each pot and, when necessary, were thinned to one plant per pot. The plants were grown in a greenhouse subjected to natural solar radiation with a temperature oscillation between 25°C and 35°C as described by Ray & Sinclair (1998) during the summers of 2005 and 2006. For control experiment, 25 pots containing the plants were watered daily with 0.5× Hoagland solution at field capacity during all the cycle of cultivation. Drought stress was applied to the remaining 25 plants, 20 d after germination until the plants displayed morphological changes caused by water deprivation, such as leaf reduction, dwarfism and premature flowering. Both control and stressed plants were collected when they were 40 d old. Photosynthetic, expanded leaves of the same age and roots were collected and frozen at –80°C for molecular biology experiments.

Quantification of photosynthetic parameters

Drought-stressed plants and controls were analysed for their content of intracellular CO_2, transpiration, photosynthesis and stomatal conductivity using an infrared gas analyser (IRGA Li-6400; Li-COR, Lincoln NE, USA). The measurements were performed at constant temperature of 30°C and a constant irradiation of 1000 mmol $m^{2\ -1}s^{-1}$. The values were normalized with the foliar area in each case of trifoliate, fully expanded leaves. Leaves were excised from both treatments and used to calculate their relative water content (RWC), as described by Barrs & Weatheley (1968) and Barrera-Figueroa *et al.* (2007). A RWC of 70–80% was considered early, 50–70 intermediate and < 50, severe drought.

Construction of a cDNA suppressive–subtractive library (SSL)

RNA from leaves and roots of 'Pinto Villa' and 'Carioca' was extracted as in Logemann *et al.* (1987), mRNA was then purified using a commercial system (Oligotex mRNA kit; Qiagen Hilden, Germany). The SSL was constructed as described by Diatchenko *et al.* (1996), using the PCR (polymerase chain reaction)-Select cDNA Subtraction Kit (BD Biosciences Clontech, Mountain View, CA, USA). cDNA from water-stressed 'Pinto Villa' plants was used as driver, while cDNA from water-stressed 'Carioca' was used as tester. Polymerase chain reaction products from the second amplification were cloned into the pDRIVE vector (Qiagen). Recombinant plasmids harboring subtracted cDNA from 'Pinto Villa' were analysed by restriction; from the library, 500 independent clones were sequenced at Langebio, Cinvestav Irapuato, México, with an ABI PRISM 6000 genetic analyser (Applera Corp., Norwalk, CT, USA) using the dideoxy method and categorized by the WV-BLAST 2.0 program of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). DNA sequence of the 500 clones was compared with the GenBank using BLASTN and BLASTP comparisons.

Quantification of differential expression using a macroarray

Recombinant plasmids containing different gene-encoding proteins were propagated and normalized to 250 ng μl^{-1} and then denatured with 0.2 N NaOH (final concentration) for 10 min. Plasmids were blotted by duplicate onto positive-charged Nylon membranes and fixed with UV radiation (4 uJ min^{-1}) with the UV Crosslinker (Stratagene, La Jolla CA, USA). Membranes where then washed three times with 2× standard saline citrate (SSC), 0.5% sodium dodecyl sulfate (SDS) and hybridized with the ^{32}P-cDNA probes. RNA extracted from stressed and normal leaves or roots was used as template to synthesized cDNA using oligo dT-GAGA primer (Invitrogen, Carlsbad, CA, USA) in the presence of ^{32}P-dCTP. Nylon membranes containing the recombinant collection were hybridized with ^{32}P-cDNA synthesized from drought-stressed or control leaves or roots mRNA. As control for loading, the 26S RNA-encoding gene was employed. Hybridization and washes were performed as described by Barrera-Figueroa *et al.* (2007) and the signal intensity was quantified with the software ARRAYVISION 7.0 (GE Healthcare, Piscataway, NJ, USA). The values obtained were normalized with the 26S RNA signal.

mRNA in situ hybridization

Plant tissue was collected and processed as described by Ruiz-Medrano *et al.* (1999). Tissue collected included fully developed, photosynthetically active leaves. Trifoliated branches of the same position were sampled in both cultivars. Roots were excised and dissected from the distal part at the same length from the emerging root. Excised tissue was placed in paraffin blocks and sectioned with a microtome HM 315 (Microm, Walldorf, Germany). Sense and antisense RNA probes were synthesized by *in vitro* transcription using the MAXIscript kit (Ambion, Austin TX, USA) in the presence of digoxigenin-UTP, following the manufacturer's instructions. The plasmid AqpH-13, a pDRIVE T-vector (Qiagen) containing the AQP-encoding open reading frame (ORF) was used as template. This plasmid bears the AQP ORF, which was cloned as a PCR fragment. The orientation was analysed by sequencing, thus, the SP6 promoter drives the transcription of the sense RNA, while the T7 promoter drives the transcription of the antisense RNA. Linear plasmids, digested with *Nco*I or *Pst*I were used for the synthesis of sense and antisense RNA, respectively. Detection of labeled mRNA was performed with antidigoxigenin antibodies conjugated with alkaline phosphatase (AP). The AP activity was then assayed in the presence of NBT-BCIP (Nitroblue tetrazolio/bromo-chlorine-indol phosphate; Roche, Basel, Switzerland). *In situ* hybridized tissue was photographed with a digital camera adapted to an HFX-DX microscope (Nikon) for further analysis.

Northern blot

Total RNA was isolated from leaf, apices, stem, petiole and root of each cultivar subjected to watered and drought conditions. A 10-µg sample of this RNA was loaded per lane, blotted onto a nylon membrane, UV-crosslinked and hybridized against an AQP probe basically as described by Sambrook & Russell (2001). A ^{32}P-labeled probe was generated by random priming (Perkin-Elmer, Wellesley, MA, USA). Hybridization was performed under stringent conditions, using the Church buffer (1% bovine serum albumin (BSA), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 M $NaPO_4$, pH 7.5, 7% sodium dodecyl sulphate (SDS)) at 65°C. Membranes were washed also under stringent conditions (1× SDS, 1× SSPE at 65°C, 30 min; SSPE, see Sambrook & Russell, 2001), after which these were exposed to X-ray films at –80°C.

Rapid amplification of cDNA ends (RACE)

The following primers were used for the amplification of a fragment of the 'Carioca' AQP cDNA: forward 5′-GGCATAGGAACCAATGAAGACATCTCC-3′ and reverse, 5′-GGTACCATTGCACCCATTGCTATTGGG-3′. These primers were used to obtain the ORF from both 'Pinto Villa' and 'Carioca' AQP cDNAs as described by Schaefer (1995), an additional primer was used to amplify the 5′ UTR (untranslated region) of the 'Pinto Villa' cDNA; forward 5′-GACGACAGAAGGGGGAGGCAAGGTAGC-3′. The amplified product was then cloned into the pDRIVE T-vector (Qiagen) for further analysis.

Phylogenetic analysis

The deduced amino acid sequences from AQPs used in this analysis were obtained from the GenBank database, and were selected to include representative organisms belonging to different kingdoms. Sequences from glycerolporins or ammonium transporters were not included in this analysis. The identification of motifs was performed using the database CDD (Conserved Domain Database) for protein classification (Marchler-Bauer *et al.*, 2005); sequence comparison was performed using the CLUSTAL X program (Thompson *et al.*, 1997) with default settings. The species name is indicated in the phylogenetic trees, accession numbers of the corresponding species are indicated in the Table 1. The alignments were then edited with the SEAVIEW program (Galtier *et al.*, 1996). Phylogenetic estimations were calculated using PAUP 4.0b10 for 32-bit Microsoft Windows (D.L.S. Swofford, Sinauer Associates, Inc., Sunderland, MA, USA). Finally, a heuristic search was performed with 100 random stepwise addition replicates. A maximum of 1000 trees for each replicate were performed. Successive approximations were performed on data sets weighted a posteriori based on the rescaled consistency index derived from trees obtained from unweighted parsimony analyses. A total of 1000 replicates bootstrap was done, and a consensus tree was obtained using the majority rule. The branches with less than 50% bootstrap support were collapsed in the consensus tree.

Results

Behavior of 'Pinto Villa' and 'Carioca' under drought stress

Fifty plants of both cultivars were grown for 20 d under irrigation at field capacity and drought stress was applied to 25 plants by eliminating the watering. Both control and stressed plants were maintained in the greenhouse. After 20 d of drought, the relative water content was calculated from leaves from 'Pinto Villa' and 'Carioca' under normal irrigation and drought stress. Irrigated 'Pinto Villa' and 'Carioca' contained a RWC of 83% and 89%, respectively (Table 2). 'Pinto Villa' and 'Carioca' under drought conditions contained a RWC of 70% and 68%, respectively, which is considered intermediate

Table 1 List of aquaporin sequences used to construct the phylogenetic tree

Tonoplast aquaporins
Triticum aestivum, AY525639; *T. aestivum,* AY525640; *T. aestivum,* AY525639; *T. aestivum,* AJ843991; *Spinacia oleracea,* AJ245953; *Arabidopsis thaliana,* AJ605573; *Vitis vinifera,* AY839872; *Posidonia oceanica,* AJ314583; *Nicotiana tabacum,* Y08161; *Brassica napus,* AF118381; *A. thaliana,* AY059134; *A. thaliana,* AF370172
nodulation (NOD)-like
Toxoplasma gondii, AJ581909; *Nicotiana alata,* U20490; *A. thaliana,* Q9C6T0; *A. thaliana,* Y07625; *A. thaliana,* AJ276475. *Drosophila melanogaster,* NP523697; *Oryza sativa,* AAL99713; *Leishmania mexicana,* AY845217; *Homo sapiens,* NM004925
Plasma membrane
Mesembryanthemum crystallinum L36097; *V. vinifera,* AF188843; *S. oleracea,* AJ249384; *V. vinifera,* AF188844; *Plantago major,* AJ843992; *Brassica rapa,* AF004293; *A. thaliana,* AY063931; *A. thaliana,* AY091254; *A. thaliana,* AY048294; *Vicia faba,* AF266760; *Vicia faba,* AJ289701; Lycop*ersicon esculentum,* Q08451; *N. tabacum,* AF440271; *P. oceanica,* AJ314584; *S. oleracea,* AY372191; *Medicago sativa,* AF004708; *Ricinus communis,* AJ605574; *Phaseolus vulgaris,* AAY22203; *Vitis vinifera,* AY823263; *B. napus,* AF118383; *A. thaliana,* AY064019; *A. thaliana,* AY096701; *A. thaliana,* AF370351; *A. thaliana,* AY062981; *A. thaliana,* BT000670; *A. thaliana,* AAL09798; *A. thaliana,* AAL06803; *A. thaliana,* AY045690; *R. communis,* AJ605575; *N. tabacum,* AF440272; *S. oleracea,* L77969; *Sus scrofa,* NP999619; *Mus musculus,* NP031498; *Rattus norvegicus,* CAA49761; *Pongo pygmaeus,* CAH92091; *H. sapiens,* AY953319; *H. sapiens,* AAB31193; *H. sapiens,* AAH22486; *H. sapiens,* NM001651; *H. sapiens,* NM001650

Table 2 Comparison of physiological parameters of irrigated and drought-stressed common bean *Phaseolus vulgaris* 'Pinto Villa' and 'Carioca'

	'Pinto Villa' (tolerant)		'Carioca' (susceptible)	
	Irrigated	Drought	Irrigated	Drought
Relative water content (RWC)[a]	83 ± 4.2	70 ± 5.8	89 ± 3.3	68 ± 4.3
Intracellular CO_2 (iC, μmol CO_2 mol^{-1} air)	75.5 ± 2.1	0.0021 ± 0.001	43.5 ± 3.2	209 ± 12
Transpiration (mmol H_2O m^{-2} s^{-1})	1.54 ± 0.1	0.19 ± 0.09	3.56 ± 0.7	0.14 ± 0.08
Photosynthesis (μmol CO_2 m^{-2} s^{-1})	9.96 ± 0.28	2.63 ± 0.3	15.3 ± 2.2	0.54 ± 0.13
Conductivity (mol H_2O m^{-2} s^{-1})	0.046 ± 0.002	0.003 ± 0.0003	0.073 ± 0.01	0.024 ± 0.001

Three measurements were performed for each condition at constant temperature (30°C) and constant irradiation (1500 μmol m^{-2} s^{-1}). Five plants were used for each treatment.
[a]RWC (%) = ((FW – DW)/(TW – DW)) × 100. FW, DW and TW are fresh, dry and turgid weight, respectively (Barrs & Weatheley, 1968). RWC in irrigated soil, 90%; under drought conditions, 15%.

stress. 'Pinto Villa' plants displayed a reduction in the foliar area, while 'Carioca' plants displayed morphological changes such as reduction of foliar area, dwarfism and premature flowering. Physiological parameters indicated that under watering conditions, 'Carioca' had higher photosynthetic activity and transpiration rate when compared with 'Pinto Villa', but similar intracellular CO_2 (iCO_2) and stomatal conductivity (Table 2). By contrast, drought-stressed 'Carioca' plants displayed a high concentration of intracellular CO_2, compared with 'Pinto Villa', presumably owing to the combination of stomata closure and the exaggerated reduction in its photosynthetic activity (3.2% of its rate under normal conditions). Despite the stress conditions, 'Pinto Villa' registered a detectable photosynthetic activity (26%), decreased iCO_2 concentration and higher transpiration rate, with similar stomatal conductivity (Table 2).

Soil RWC was calculated in 90% and 15% in watered and intermediate drought stress, respectively; this is equivalent to the drought stress expected in field conditions. When severe stress (RWC < 50%) was obtained in both plants, 'Carioca' plants reached their point of permanent wilting, since after irrigation under those severe conditions, no plant recovery was observed, by contrast to the normal recovery of 'Pinto Villa' under the same stress.

Construction of the subtractive library and identification of enriched cDNAs in the drought-tolerant variety Pinto Villa

It was assumed that differential expression of genes as a response to water deficit would be present in both aerial and root tissues; thus, RNA was extracted from these tissues from 'Pinto Villa' and 'Carioca' plants subjected to drought stress for 20 d. cDNA was synthesized and a SSL was constructed (as described in the Materials and Methods section) by subtracting 'Carioca' cDNA from 'Pinto Villa' cDNA. Five-hundred independent cDNAs from this library were sequenced and compared with the database to classify them by function. Two macroarrays with the plasmids blotted by duplicate were constructed and tested with each contrasting ^{32}P-cDNA, as described in the Materials and Methods section. The upregulation was quantified by comparing the signal intensity obtained from both macroarrays, normalized with the signal obtained with the 26S RNA (positive control). The value obtained for upregulation is listed in Table 3. A number of genes already described as associated to drought stress were identified; those included a TIP with a redundancy of 8.2%, and an upregulation of 1.3 and 1.1 in roots and leaves, respectively); dehydrin, a moisture-responsive hypothetical protein and an

Table 3 Functional classification of cDNAs isolated from the drought-tolerant *Phaseolus vulgaris* 'Pinto Villa' subtracted with susceptible 'Carioca' cDNAs

Clone	Similarity	Length (bp)	*E*-value	Reference	Redundancy (%)	Upregulation*	
						Roots	Leaves
Drought stress							
H08	Tonoplast intrinsic protein *Cicer arietinum*	346	1e-64	AJ630659	8.2	1.3	1.6
R04	Dehydrin (dhn1) *Picea abies*	240	1e-20	AY961924	1.1	1.5	2.4
R03	Moisture stress responsive cDNA *Retama raetam*	51	7e-06	AY725183	1.1	1.5	1.4
H10	Chloroplast small Hsp precursor *Lotus corniculatus*	174	4e-10	AP007298	1.1	1.1	2.0
Cold stress							
H12	Cold-induced *Triticum aestivum*	96	2e-11	CK168002	1.1	2.1	2.5
Signal transduction							
H02	Profilin *P. vulgaris*	148	7e-12	X81982	13	1.3	2.0
H08	Protein kinase SPK-3 *Phaseolus acutifolius*	65	6e-10	AY220097	25.1	3	1.6
Plant defense							
R06	Thaumatin-like protein *Sambucus nigra*	69	1e-06	AK060450	2.3	1.5	2.2
Chloroplast function							
H11	Carboxyl transferase a subunit accA *Glycine max*	128	2e-33	AF164510	5.2	1.0	1.6
H06	Chloroplast phosphoglucomutase *G. max*	139	4e-18	BG511176	3.5	1.1	1.9
H09	Chloroplast protein *Medicago truncatula*	207	1e-08	AC123572	7.6	1.1	1.8
Oxidative stress							
H04	Thioredoxin cdsp32 *Oryza sativa*	341	3e-04	AM039890	1.1	1.2	2.0
Unknown function							
R05	Hypothetical protein *M. truncatula*	286	1e-09	CR931735	1.1	3.5	3.2
R04	Hypothetical protein *M. truncatula*	182	0.023	AC146649	7.6	1.3	2.0
H07	*Phaseolus vulgaris* est	69	1e-20	CV533172	1.7	2.4	1.6
H09	Hypothetical protein *O. sativa*	139	2e-14	AP008215	0.6	2.0	2.5
R02	Hypothetical protein *O. sativa*	212	2e-20	XM_470182	5.8	1.1	1.9
R10	Hypothetical protein *Arabidopsis thaliana*	39	1e-03	AF296834	0.6	1.2	2.6

Hsp precursor from *Lotus corniculatus*. A cold stress-induced protein from *Triticum aestivum* was also found. Signal transduction associated proteins were also identified, such as profilin, protein kinase SPK-3 (which was the most redundant cDNA (25%), suggesting that stress perception is mediated by phosphorylation events. Additional proteins associated with chloroplast function, such as carboxyl transferase α subunit, and a phosphoglucomutase strongly suggests the photosynthetic activity is maintained in the tolerant variety vs the susceptible plant. A thioredoxin was also found, thus indicating the close relation already studies of drought with oxidative stress. Finally, several cDNAs similar to sequences with unknown function were found in the library. Some of these cDNAs have been previously associated with the response to drought stress. The TIP cDNA was selected for further analysis in terms of its regulation under drought stress as well as for its redundancy in the subtractive library.

Characterization of AQP mRNAs in drought-tolerant and -susceptible common bean cultivars in response to water deficit

From a set of 500 cDNA clones, 8.2% of these corresponded to a putative AQP. According to a BLAST search, the truncated sequence obtained from the library corresponded to a tonoplast AQP (see below). The clone from the subtractive library contained 400 bp in length, encompassing 150 bp of the 3′ UTR and 250 bp of the 3′ portion of the ORF. In order to obtain the full-length cDNA, RACE for the 5′ portion was performed as described. In parallel, using primers specific for the 'Pinto Villa' AQP were used to amplify the 3′ portion of the ORF fragment of the 'Carioca' AQP homolog. This was also cloned and sequenced, and found to have extensive similarity to the 'Pinto Villa' fragment. Indeed, both were identical at the amino acid level. A 5′ RACE was performed for these cDNAs, using stem, leaf, petiole, root and phloem sap exudate cDNAs from plants grown in the contrasting conditions. Finally, using specific primers, the ORFs from both AQPs were cloned, sequenced, and it was found that, at the amino acid level, they are identical. However, there are a few silent substitutions in the DNA sequence. Interestingly, their 5′ UTRs are 100% homologous throughout 90 bases. The ORFs identified are 234 codons in length, encoding a protein of theoretical molecular mass of 26.2 kDa. 'Pinto Villa' AQP (GenBank accession number DQ087217) and 'Carioca' AQP (DQ087218) inferred amino acid sequences were then compared with representative members of the AQP family present in common bean plants. Figure 1 gives a comparison of the deduced amino acid sequence of putative tonoplast AQPs from 'Pinto Villa' and 'Carioca' with TIPs (CAA44669, P23958, JQ1106, PQ0185; Johnson *et al.*, 1990) and MIPs (AAY22204, AAB72149, AAY22203; F. Campos *et al.* unpublished; R. Aroca, unpublished). Short conserved regions are present throughout the length of the sequence. As a conserved signature of the family, a motif represented by the amino acids asparagine, proline and alanine (NPA) is repeated twice. These motifs, which are part of the active site, are located in the central region of the molecule and separated by 100 residues (± 9). The three-dimensional structures of several AQPs have been obtained (Scheuring *et al.*, 1999; Schenk *et al.*, 2005). A simulation of the AQP three-dimensional structure shows a striking resemblance to the resolved structure of the *Escherichia coli* AQP (data not shown), confirming the evolutionary conservation of these proteins. Phylogenetic analysis of AQPs also supports the assumption that both 'Pinto Villa' and 'Carioca' AQPs reside in the tonoplast (Fig. 2). The closest homology of 'Pinto Villa' and 'Carioca' was found with the TIP sequence accession number PQ0185 from kidney bean, which was calculated in 58%.

Common bean AQPs are part of a diverse gene family

In order to determine the similarity of the common bean AQP with other members of this family, a phylogenetic tree was calculated. The computed unrooted phylogenetic tree is divided into two major groups, and has a similar topology to the one described by El Karkouri *et al.* (2005), considering that channels for small organic molecules were not included in the present analysis. The first group (supported by a 97 bootstrap value) is formed by AQP sequences that correspond exclusively to plant proteins from several species; 19 of these have been experimentally shown to reside in the plasma membrane (according to the information provided by GenBank). This group is subdivided into two groups, although the functional basis for such divergence is not clear. The second major group (with a 95 bootstrap value) is rather heterogeneous since, within this clade, there are at least three identified subgroups. These include animal and plant nodulation (NOD)-like and plant tonoplast proteins. Interestingly, a small branch includes sequences from animal (human and *Drosophila*) and Apicomplexa. The clustering of these in a single group (with a rather low bootstrap value) could be an artifact of few available sequences from Apicomplexa. This clade also includes a drought inducible AQP from *Oryza sativa*, which has not been characterized further. The plant AQP forms at least two discrete clades. The smallest one (95 bootstrap percentage) is formed by three *Arabidopsis thaliana* NOD-like AQPs and a pollen-specific protein from *Nicotiana alata*. This last one corresponds to a plasma membrane protein. Another clade (69 bootstrap value) in this large heterogeneous group is formed by 16 sequences, including the 'Pinto Villa' and 'Carioca' AQPs. In this clade 11 proteins have been found to localize to the tonoplast, whereas five sequences from *Plantago major*, *A. thaliana* and *P. vulgaris* have not been characterized and thus their localization has not been established. However, the grouping of *P. vulgaris* AQPs strongly suggest that their localization is confined to the tonoplast membrane. It must be

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P.v. Pinto Villa  19 KAYFSELHATLIFVFAGVGSAIAYNELTKDA-----AldptGLVAVAVAHAFALFVGVSVAANISGGHLNPAVTFGLAVG  93
P.v. Carioca      19 KAYFSELHATLIFVFAGVGSAIAYNELTKDA-----AldptGLVAVAVAHAFALFVGVSVAANISGGHLNPAVTFGLAVG  93
E. coli            9 GQCIAEFLGTGLLIFFGVGCVAALkvag----------asfgQWEISVIWGLGVAMAIYLTAgvSGAHLNPAVTIALWLF  78
G. max            38 QKLVAEAVGTYFLIFAGCASLVVNenyy----------nmitFPGIAIVWGLVLTVLVYTVGhiSGGHFNPAVTIAFAST 107
A. majus          19 KAYVAEFIATLLFVFAGVGSAIAYnkltsda-----aldpagLVAVAVAHAFALFVGVSMAAnvSGGHLNPAVTLGLAVG  93
A. canescens      40 RAAIAEFIATLLFLYITVATVIGYkketdp-------casvgLLGIAWSFGGMIFVLVYCTAgiSGGHINPAVTFGLFLA 112
A. thaliana       24 RATLAEFLSTFVFVFAAEGSILSLdklywehaahagtntpggLILVALAHAFALFAAVSAAInvSGGHVNPAVTFGALVG 103
man               12 RAVVAEFLATTLFVFISIGSALGFkypvgnn-----qtavqdNVKVSLAFGLSIATLAQSVGhiSGAHLNPAVTLGLLLS  86
consensus          1 RKYLAEFLGTFLLVFFGCGSVLAVKLAGGAS-----G----GLLGIALAWGFAIFVLVYAVGHISGGHINPAVTLALAVG  71

P.v. Pinto Villa  94 GNITLLTGFLYWIAQLLGSILACLLLNLVT-------------AKS---------IPTHGPAYGVNAFQGVVCEIIITFG 151
P.v. Carioca      94 GNITLLTGFLYWIAQLLGSILACLLLNLVT-------------AKS---------IPTHGPAYGVNAFQGVVCEIIITFG 151
E. coli           79 ACFDKRKVIPFIVSQVAGAFCAAALVYGLYYNLffdfeqthhivrgsvesvdlagtfstypnphinfVQAFAVEMVITAI 158
G. max           108 RRFPLIQVPAYVVAQLLGSILASGTLRLLFMGNhdqfsgt---------------------vpngtnLQAFVFEFIMTFF 166
A. majus          94 GNITILTGLFYWIAQCLGSTVACLLLKFVTNGLsvpthgv---------------------aagmdaIQGVVMEIIITFA 152
A. canescens     113 RKVSLLRALVYMIAQCAGAICGVGLVKAFMKGPynqfgggan-----------------svalgynkGTAFGAELIGTFV 175
A. thaliana      104 GRVTAIRAIYYWIAQLLGAILACLLLRLTTNGMrpvgfrl----------------------asgvgaVNGLVLEIILTFG 162
man               87 CQISIFRALMYIIAQCVGAIVATAILSGITSSLtgnslgrn------------------dladgvnsGQGLGIEIIGTLQ 148
consensus         72 GRFPLIRVIPYIIAQLLGAILGAALLYGLYYGLyleflganniVAG---------IFGTYPSPGVSNGNAFFVEFIGTFI 142
                                                                    ***
P.v. Pinto Villa 152 LVYTVYATAADPKKG---SLGTIAPIAIGFIVGANILAAGPFSGGSMNPARSFGPAVVSGNF----------A---DNWI 215
P.v. Carioca     152 LVYTVYATAADPKKG---SLGTIAPIAIGFIVGANILAAGPFSGGSMNPARSFGPAVVSGNF----------A---DNWI 215
E. coli          159 LMGLILALTDdgngv---prgpLAPLLIGLLIAVIGasmgplTGFAMNPARDFGPKVFawlagwgnvaftggrdipYFLV 235
G. max           167 LMFVICGVATdnra-----vgeFAGIAIGSTLLLNViiggpvTGASMNPARSLGPAFVhge-------------yeGIWI 228
A. majus         153 LVYTVYATAAdpkkg---slgvIAPIAIGFIVGANIlaagpfSGGSMNPARSFGPAVAsgd-------------fsQNWI 216
A. canescens     176 LVYTVFSATDpkrsardshvpiLAPLPIGFAVFMVHlatipiTGTGINPARSFGAAVIynkk----------rvwdDHWI 245
A. thaliana      163 LVYVVYSTLIdpkrg---slgiIAPLAIGLIVGANIlvggpfSGASMNPARAFGPALVgwr-------------whDHWI 226
man              149 LVLCVLATTDrrrr----dlggSAPLAIGLSVALGHllaidyTGCGINPARSFGSAVIthn-------------fsNHWI 211
consensus        143 LVLVVFATTDDPNGP---PPGGLAPLAIGLLVAAIGLAGGPITGASMNPARSLGPALFTGLA----------RhwhYFWV 209

P.v. Pinto Villa 216 YWVgpliggglagliygdv 234
P.v. Carioca     216 YWVgpliggglagliygdv 234
E. coli          236 PLFGPIVGAIVGAFAYRKL 254
G. max           229 YLLAPVVGAIAGAWVYNIV 247
A. majus         217 YWAGPLIGGALAGFIYGDV 235
A. canescens     246 FWVGPFVGALAAAAYHQYV 264
A. thaliana      227 YWVGPFIGSALAALIYEYM 245
man              212 FWVGPFIGGALAVLIYDFI 230
consensus        210 YWVGPLIGAIAGALVYDYV 228
```

Fig. 1 Comparison of the deduced amino acid sequence of aquaporins reported in beans. GenBank accession numbers: 'Pinto Villa', DQ087217; 'Carioca', DQ087218; tonoplast intrinsic proteins (TIPs): CAA44669, P23958, JQ1106 (Johnson *et al*., 1990); membrane intrinsic proteins (MIPs): AAY22203, AAY22204 (R. Aroca *et al.* unpublished), AAB72149 (F. Campos *et al.* unpublished). Capital letters show identities or conserved substitutions, small caps stand for nonconserved amino acids. Asterisks indicate the asparagine, proline and alanine (NPA) aquaporin-conserved motifs.

stressed that the subcellular localization of AQPs is dynamic, and responds to environmental stimuli (Pao *et al*., 1991; Vera-Estrella *et al*., 2004).

Aqp1 mRNA differentially accumulates in organs of both cultivars

Analysis by Northern blot was carried out in order to determine the tissues in which the AQP transcripts of both 'Pinto Villa' and 'Carioca' accumulate. Total RNA from leaves, stems, petioles, apices and roots were extracted from plants grown under normal watering or drought conditions. Northern blot analysis shows accumulation of a 1.2 kb Aqpl mRNA in roots of drought-stressed 'Pinto Villa' plants (Fig. 3), with no detectable levels in the other tested tissues and in normal conditions. By contrast, the tropical, drought-susceptible cultivar accumulated the corresponding RNA only in leaves of watered plants. The ethidium bromide stained gel is displayed in the lower panel of each blot to show even loading of RNA per lane. It should be emphasized that the blots in both cases were exposed for similar lengths of time (3 d to 1 wk), indicating that the levels of accumulation of these transcripts are lower than for other reported AQPs from plasma membrane (Fraysse *et al*., 2005). Despite the presence of additional TIPs possibly expressed in these conditions we failed to detect them under the stringent conditions used. Reverse transcriptase polymerase chain reaction (RT-PCR) assays showed a faint signal present in all the tissues tested (data not shown), indicating that the Northern blot detected the most abundant transcript present in drought-stressed roots in 'Pinto Villa' and watered 'Carioca' leaves.

PvAqp1 mRNA accumulation is relocated in specific cell types in response to water deprivation in 'Pinto' Villa, but not in 'Carioca'. The spatial accumulation of the AQP mRNA in both 'Pinto Villa' and 'Carioca' was determined by *in situ* hybridization. Forty-day-old, fully irrigated and drought-

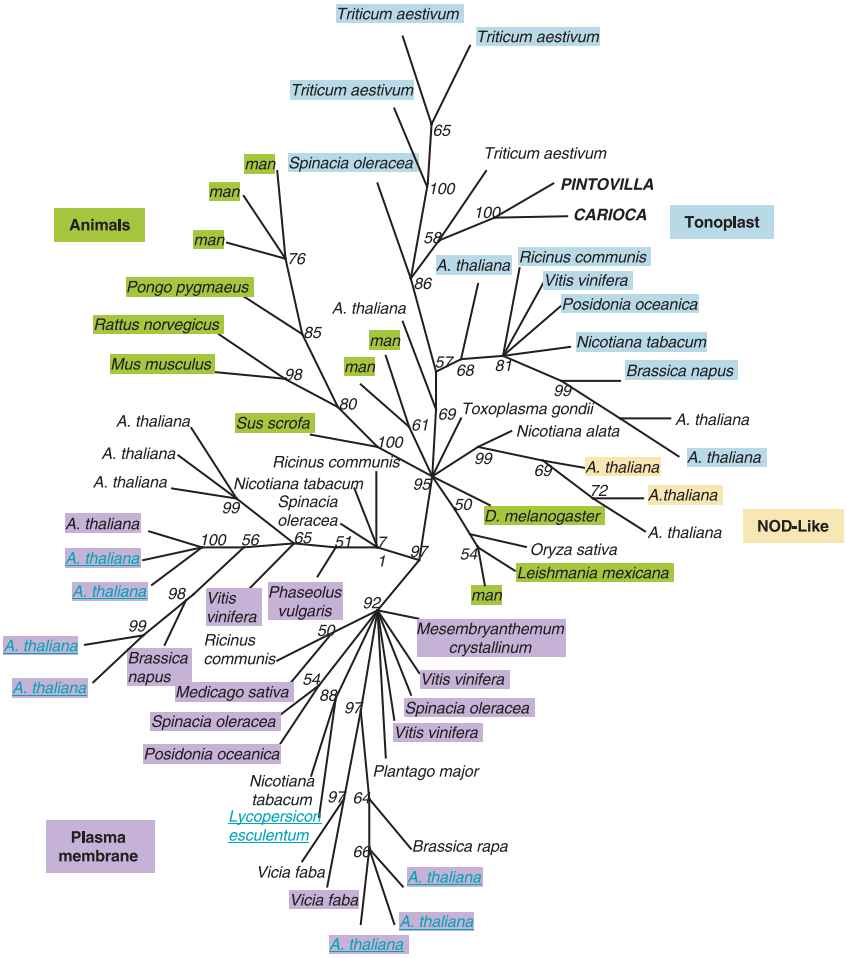

Fig. 2 Dendrogram of maximum parsimony of the aquaporin (AQP) family. Selected sequences from different eukaryotic kingdoms were compared with CLUSTAL X program. A heuristic search was carried out with 100 random stepwise addition replicates. A bootstrap analysis with a total of 1000 replicates was done, and a consensus tree was obtained using the majority rule. Numbers in the branches indicates the proportion of replicates in the random comparison. The branches with < 50% bootstrap support were collapsed in the consensus tree. The species name is indicated, accession numbers of each sequence in the GenBank database is described in Table 2. Boxed names indicate AQPs with annotation to belong to the correspondent group. NOD, nodulation.

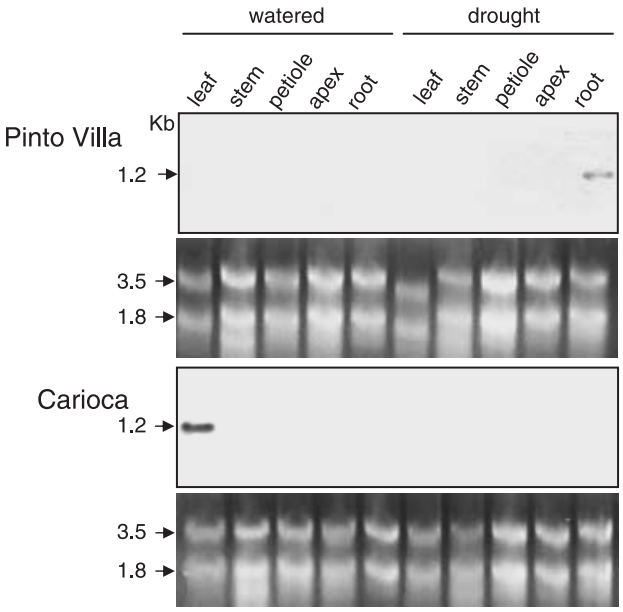

Fig. 3 Aquaporin (AQP)-encoding mRNA is differentially accumulated in both *Phaseolus vulgaris* cultivars ('Pinto Villa' and 'Carioca') under drought conditions. The expression of AQP genes in various organs was determined by Northern blot analysis; RNA loading was verified by ethidium bromide staining. Molecular size markers at the left indicate the rRNA bands. A ^{32}P-AQP open reading frame (ORF) was used as a probe and was hybridized and washed under stringent conditions (as described in the Materials and Methods section).

stressed plants (as described in the Materials and Methods section) were processed and semithin sections of leaves, stems and roots were hybridized with antisense or sense RNA. Leaves of watered 'Carioca' plants displayed an important mRNA accumulation in mesophyll, epidermis and parenchyma, as observed in cross-sections. By contrast, irrigated 'Pinto Villa' leaves only show mRNA accumulation in root cortical cells (Fig. 4, upper panel). Under drought conditions only the mRNA-associated signal was observed in 'Pinto Villa' leaf, stem and root phloem tissues, and was confined to immature (cambium) and mature phloem cells (Fig. 4, medium and lower panel). No signal was visible in all the treatments when sense RNA was used as a probe, indicating the specificity of the detection (data not shown). Interestingly, under drought conditions, the roots of 'Carioca' developed a reduced vascular system, located in the center of the root, in

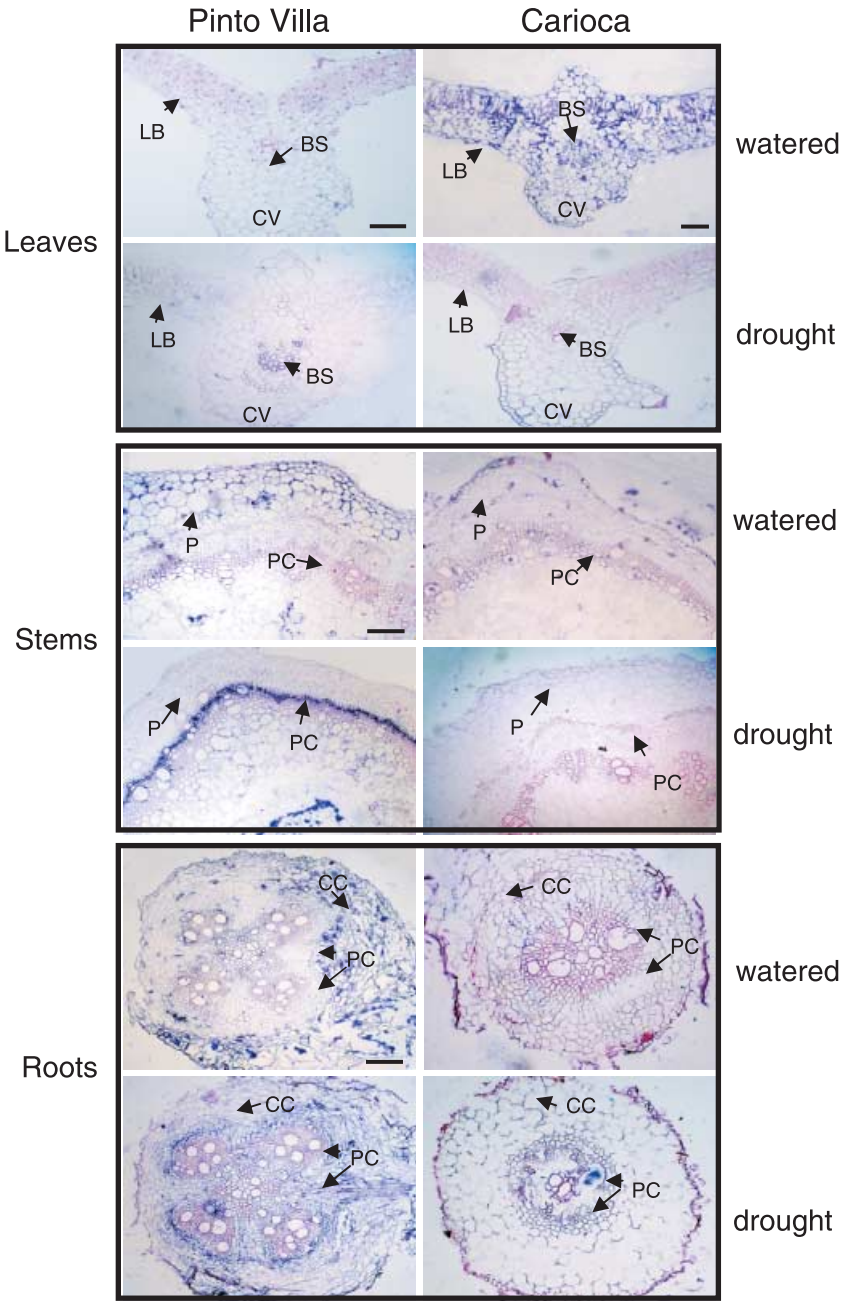

Fig. 4 Aquaporin (AQP) mRNA *in situ* hybridization in transversal sections of *Phaseolus vulgaris* 'Pinto Villa' and 'Carioca' leaves, stems and roots grown under normal and drought conditions. Tissues and treatments are indicated. Watered: 'Carioca' leaf displays the highest mRNA accumulation in mesophyll and parenchyma cells. Both cultivars bear AQP mRNA in all the cell types under irrigation, while under drought conditions, only 'Pinto Villa' display mRNA-associated signal, now restricted to the vascular cells in all the tissues tested. LB, leaf blade; CV, central vein; BS, bundle sheath; P, parenchyma; PC, phloem cells; CC, cortical cells. Bars, 100 μm.

contrast to 'Pinto Villa', in which a cruciform vascular system with conspicuous xylem was developed (Fig. 4, lower panel). In agreement with the findings in the *in situ* hybridization experiments, the RT-PCR assays performed with 'Pinto Villa' mRNA from the same tissues could detect faint bands indicating presence, although in low amounts, in stressed stems and leaves, and irrigated roots (data not shown).

Discussion

Common bean is cultivated under drought conditions in several developing countries, where drought is one of the most important factors that limit productivity. Common bean cultivars have been selected in Mexico that display tolerance to water deficit in field conditions, such as 'Pinto Villa'. 'Pinto Villa' and 'Carioca' under moderate drought conditions achieved a comparable, nonsignificantly different RWC (70% and 68%, respectively); however, while both cultivars showed a reduction in the foliar area, 'Carioca' plants displayed additional morphological changes such as dwarfism and premature flowering. When severe stress (RWC < 50%) was applied in both plants, 'Carioca' plants reached their point of permanent wilting, since after irrigation under those severe conditions, no plant recovery was observed, in contrast to the

normal recovery of 'Pinto Villa' under the same stress. 'Pinto Villa' is remarkable not only because of its tolerance to drought, but also for its high yields in these conditions (*c.* 70% of the yield in full irrigation). Under normal irrigation, 'Carioca' plants displayed a high photosynthetic rate, which was severely reduced when drought stress was applied. Interestingly, 'Pinto Villa' could maintain, even though at low level, its photosynthetic capacity. The mechanisms by which this variety can resist this stress condition are not known, but several can be proposed to be important; molecular and physiological evidence presented here indicates that protection of the photosynthetic machinery and differential water mobilization could be important in explaining such tolerance. The upregulated AQP and dehydrin encoding transcripts are directly related to water use. Their function positively impacts on water use by allowing the maintenance of intracellular water potential for basic functions. In particular, the tonoplast AQP would allow the vascular system to remain functional, thus, by translocating photoassimilates to sink tissues and allowing the plant growth. Regarding the upregulation of the SPK-3 protein kinase (the most abundant upregulated transcript), which has been described as necessary for induction of abscisic acid (ABA)-responsive genes and judging by its role in signal transduction, it is provocative to assign it an important role in the activation of proteins at post-translational level, by activating via phosphorylation yet unknown proteins. The chloroplast small Hsp and the chloroplast thioredoxin, could have an important role in the maintenance of the photosystems under stress; indeed, 'Pinto Villa' reduced its photosynthesis to 26% under drought stress, while 'Carioca' showed a decrease to only 3.5% of its normal capacity under normal irrigation. Finally, the induction of profilin in response to water deficit has not been reported in other biological systems; however, given its role in cytoskeleton regulation, its role in maintaining cell shape could be important in coping with water stress. The characterization of a group 3 late embryogenesis protein (Barrera-Figueroa *et al.*, 2007) in 'Pinto Villa' indicated a rapid induction under drought conditions, however, this mRNA was not identified in the library, thus suggesting that 'Carioca' does respond to drought stress using similar gene-encoding late embriogenesis abundant (LEA) proteins. By contrast, the upregulated AQP, accumulates differentially in root cell types of the tolerant cultivar. Exactly the opposite situation was found in leaves, in which no AQP transcript was detectable in 'Pinto Villa', compared with 'Carioca', under full irrigation. However, as indicated by Northern blot analysis, the transcript accumulation in 'Carioca' is higher in drought-stressed leaves than in 'Pinto Villa'. A function in water conservation in the conducting tissue, mostly in roots, could be invoked for this particular AQP, and it may contribute, among other drought-related proteins, to the contrasting phenotypes of both cultivars under drought and watered conditions. Interestingly, the overexpression of tonoplast AQPs in *Arabidopsis* had beneficial effects on salt-stress tolerance, as indicated by superior growth status and seed germination (Peng *et al.*, 2007), suggesting a similar role for drought protection in this tolerant cultivar. The expression profiles of these mRNAs are so contrasting that would be interesting to screen different varieties and relate its spatial accumulation with drought tolerance. Both AQPs display the same deduced amino acid sequence, suggesting a conserved role, but also that the adaptation of 'Pinto Villa' to drought conditions could have occurred recently in evolution. Thus, substitutions between both genes should be present in noncoding regions such as the promoter and, or 5′ and 3′ UTRs. Significant homologies were found when these AQPs were compared with those already reported in common beans. The phylogenetic analysis presented in this work, obtained by maximum parsimony, placed the AQP from 'Pinto Villa' and 'Carioca' in the same clade of a group of eukaryotic tonoplast AQPs. Indeed, a highly homologous *A. thaliana* AQP is involved in ammonium transport in vacuoles, probably equilibrating urea concentrations between cellular compartments. Thus, in addition to its likely function as water channel, the AQP described may also be involved in the transport of other solutes. However, the assigned subcellular localization of this AQP to tonoplast by sequence comparison has to be confirmed experimentally. The construction of this phylogenetic tree is a useful tool to theoretically assign a subcellular location of other AQPs by comparing its deduced amino acid sequence. The localization of the 'Pinto Villa' and 'Carioca' AQP mRNAs also indicated that these are differentially regulated, and support the notion that *in situ* localization of transcripts and proteins may be helpful in assigning a particular role during a stimulus. Indeed, AQP mRNA accumulated to highest levels in mesophyll in 'Carioca', while in 'Pinto Villa' no signal was detected. At the macroscopic level, the leaves of 'Carioca' are pubescent, while those of 'Pinto Villa' are thinner, indicating in an indirect way the amount of water contained in that organ, which in turn can be attributed, at least in part, to the AQPs. When drought stress was applied to both cultivars, AQP mRNA accumulation in 'Carioca' decreased significantly while some signal was detectable in Pinto, albeit restricted to the phloem. The increase of tonoplast water permeability by expression of this particular AQP in the vascular system, specifically in the root phloem, may allow more efficient water conservation during dehydration stress, and allow the movement to aerial tissues. This fact indicates the systemic nature of stress tolerance, indeed, *in situ* RNA hybridization of Hsp- and dehydrin-encoding genes indicate an important localization in vascular tissue (data not shown). It must be emphasized that while the global concentrations of AQP mRNA are still higher in stressed leaves from 'Carioca' than those from 'Pinto Villa', the failure to induce such a gene in the phloem may render 'Carioca' more susceptible to water deficit. In stem and roots, again a similar pattern of AQP accumulation was observed in which the induction of AQP appears to be restricted to the phloem

in the tolerant variety. This strategy would allow the plant to maintain the integrity of the vascular system, allowing the transport of water, minerals, photoassimilates, and long-distance signaling, as described in nonstressed plants (Ruiz-Medrano *et al.*, 1999; Xoconostle-Cázares *et al.*, 1999). The resulting homeostasis allows the plant to complete its cycle, leading to seed production. No signal was detected either in developing xylem as a consequence of water deprivation; however, a significant signal was detected in the cambium and immature phloem cells in the vascular boundary, indicating that these undifferentiated vascular cell types are already compromised to synthesize AQP. This in turn suggests that the adaptation to drought is an early event in cell differentiation. The tolerance to drought stress is a quantitative trait (Seki *et al.*, 2003) in which the differential regulation of AQP genes seems to be important. The biological function of another set of genes associated with drought tolerance is currently being investigated. Together, these results will allow us to understand the physiological adaptations that highly drought-tolerant plant display both in laboratory conditions and in the field.

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